THE NON-GENOMIC SIGNALING PATHWAYS MEDIATED BY G-PROTEIN COUPLED ESTROGEN RECEPTOR 1 (GPER) IN CORONARY ARTERIES

A Dissertation

by

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ABSTRACT

Coronary heart disease (CHD) remains the leading cause of death throughout the world, and postmenopausal women are at particularly high risk for CHD. A promising new avenue of study is the novel G protein-coupled estrogen receptor (GPER) which mediates estrogen action. The major purpose of my studies in this dissertation is to investigate the role of GPER in porcine coronary artery tone regulation. In a series of studies, we tested four hypotheses: *1)* activation of GPER regulates coronary artery tone by paradoxically inducing relaxation and potentiating contraction; *2)* activation of GPER induces coronary artery relaxation by Gs/cAMP-dependent pathway(s); *3)* activation of GPER induces coronary artery relaxation via inhibition of RhoA/Rho kinase pathway by cAMP downstream targets: the exchange proteins directly activated by cAMP (Epac) as well as PKA; and *4)* activation of GPER potentiates coronary artery contraction by a Gβγ/EGFR-dependent pathway. Isometric tension studies were performed on endothelium-denuded porcine coronary arteries to test the function role of GPER and its signaling pathways. RT-PCR, Western blots, patch-clamp experiments and kinase activity assays also were employed in these studies to confirm the expression and phosphorylation of subjective proteins, channel activities and kinase activities in porcine and human coronary artery smooth muscle cells (SMCs) and coronary artery tissues. Results from these studies suggest: *1)* GPER is expressed in porcine and human coronary artery SMCs; *2)* GPER mediated coronary artery relaxation is NO-independent and involves BK_{Ca} channel activity; *3)* activation of GPER stimulates the production of

cAMP, thus activates its downstream targets PKA and Epac; *4)* GPER mediates coronary relaxation through activation of MLCP via inhibition of RhoA activity by both PKA and Epac; *5)* the interaction between AKAP and PKA is involved in the cAMP/PKA signaling mediated by GPER in coronary artery; and *6)* GPER potentiates coronary artery contraction via Gβγ signaling to stimulate transactivation of EGFR and activation of ERK1/2. These findings provided evidence of the dual effects of GPER in coronary regulation, which may help reveal the controversial actions of estrogen and provide a molecular basis for developing new compounds that better target estrogen signaling for a variety of clinical applications.

DEDICATION

To my husband, Feng Wan, for supporting me in the pursuit of higher education. To our precious daughter, Esther, for bringing millions of joys to our family and cheering me up when I was down. To my parents and parents-in-law, for unconditional giving to support our abroad study and life.

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CHAPTER I

INTRODUCTION

1.1 INTRODUCTION

"While I was going through menopause, I was also going through a divorce," said my Aunt Jane. Her overwhelming physical and emotional problems caused her to lose interest in her family and her job, leading even to an attempted suicide. After her children forced her to see a doctor, Jane was diagnosed as having severe menopausal depression and was immediately placed on hormone replacement therapy. As a result of her supplemental female sex hormones, Jane no longer suffers from menopausal symptoms and her quality of life has been restored; however, unfortunately, her marriage could not recover.

My Aunt Jane is not alone! According to statistics generated from the Women's Health Initiative trial, all women ages 45 to 55 in the U.S. are suffering from a variety of menopausal symptoms, to some extent. As estrogen levels decline in menopause there is an increase in long-term serious health risks – most notably an increased incidence of coronary artery disease. The findings of my studies will help better understand and harness the therapeutic potential of estrogen and provide the many health benefits of estrogen while limiting the potentially dangerous side effects, therefore improving the quality of life for thousands of women.

1.2 ESTROGEN

1.2.1 Estrogens and classical receptors

There are three major naturally occurring estrogens in females: estrone (E1), estradiol (E2) and estriol (E3). E2 is mainly produced in the ovaries and is most potent and predominant. Estrogenically weak estrone (E1) and estriol (E3) must be converted to estradiol (E2) to show full estrogenic actions. Estrogens circulate in the bloodstream and bind to their receptors, affecting not only uterus and female breasts, but also heart, brain, bone, liver and other targeted tissues. Classically, there are two different forms of estrogen receptors (ER): ERα and ERβ which are encoded by ESR1 (70) and ESR2 (150) respectively in human. Both estrogen receptors are widely expressed in many tissues.

The effects of estrogens are mediated by either non-genomic/rapid mechanism which happens within minutes of cell stimulations, or genomic mechanism that occurs in hours (83, 138) (Fig. 1). Lipophilic estrogens freely pass through lipid bilayer membrane of cells and bind to estrogen receptors. The ligand-receptor complex goes through nuclear membrane, specifically binds to estrogen response element (ERE), a specific DNA sequence, and promotes the gene transcription. Additionally, plasma membrane localized estrogen receptors elicit the rapid response by activating 2nd messengers, stimulating downstreamphosphatidylinositol-3(PI3K)/Akt, protein kinase C (PKC) and/or mitogen activated protein kinase (MAPK) and initiating physiological effects. Even in the absence of ligands, nuclear receptors also can be stimulated by growth factor such as epidermal growth factor (EGF) and mediate genomic effects (83).

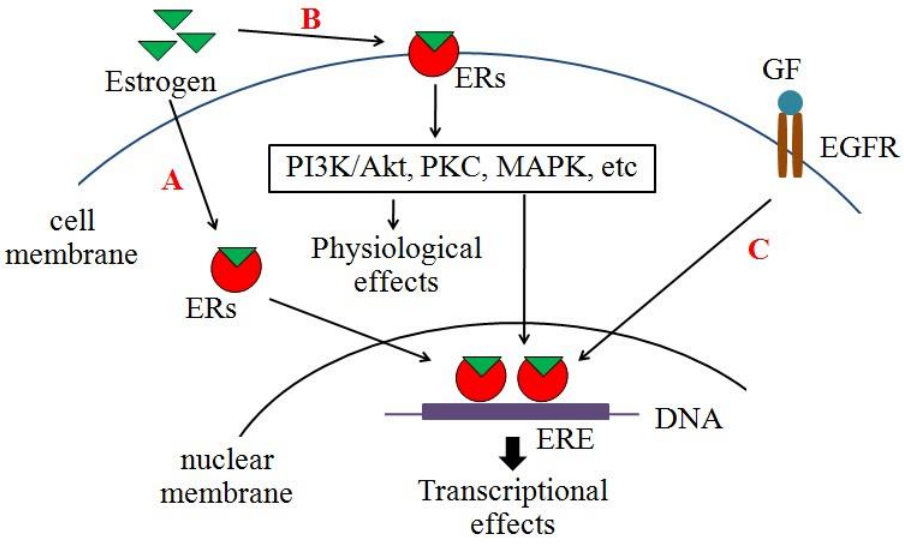

Figure 1. Genomic and non-genomic mechanisms of estrogen. A. genomic mechanism. B. non-genomic mechanism. C. ligands-independent pathway. ERs: estrogen receptors. ERE: estrogen response element. GF: growth factor. EGFR: epidermal growth factor receptor. Adapted from Ref. 153.

1.2.2 Estrogen actions on reproductive system

Estrogens present in female bodies in significantly high amounts from puberty until menopause and the primary function of estrogens is to promote development and maintenance of female secondary sex characteristics and reproduction. Generally, estrogens promote formation of the female reproductive tract, development of genitalia and breasts, distribution of female fat, growth of follicle, uterus preparation for implantation and stimulation of myometrial excitability (229). In breast tissue, for example, estrogens induce the proliferation of mammary gland cells and prepare the breasts ready for milk production during pregnancy. Estrogens also stimulate the proliferation of the endometrium of the uterus and prepare the uterus for implantation of

an embryo. After an embryo is implanted, estrogens work in coordination with progestins and other fetal hormones to control the differentiation of the fetus.

1.2.3 Estrogen actions on vascular system

Estrogens bind to their receptors on vascular endothelial cells, vascular smooth muscle cells and myocardial cells (71), eliciting physiological effects. Estrogens alter the progress of vascular diseases by influencing vasoactive molecules, proliferation of endothelium and smooth muscle cells, serum lipids and coagulation factors.

Estrogen influence on endothelial function

Endothelium, previously thought as only a single thin layer of cells lining the interior wall of blood vessels, is now proven to be involved in many aspects of vascular biology. Endothelium regulates vascular tone by producing and releasing various endothelium-derived vasoactive factors. Estrogens activate nitric oxide synthase and prostacyclin synthase subsequently increasing the production of nitric oxide and prostacyclin, which induce vasodilation immediately after estrogens are administered. Long-term administration of estrogens increase expression of genes for both vasodilatory enzymes nitric oxide synthase and prostacyclin synthase (18), and decrease the plasma concentrations of vasoconstrictors renin and endothelin-1 (26). Estrogens also accelerate the proliferation of endothelial cells, inhibit apoptosis of endothelial cells (206) and inhibit the growth of smooth muscle cells, thereby, contributing to vascular protection against vascular injury and atherosclerosis.

Estrogen influence on the serum lipids

It is well accepted that the development of atherosclerosis is directly related to the elevated level of the oxidation of low-density lipoprotein (LDL). Oxidized LDL functions as an atherogenic factor through impairing endothelium-dependent relaxation and a chemotactic factor which stimulates circulating monocytes toward the lesion site of a vessel (35). In addition, macrophages uptake oxidized LDL and accumulate foam cells in the intima, which is the first step in the development of atherosclersosis. Oppositely, HDL inhibit and protect against LDL oxidation, reversing the formation of atherosclerosis lesion (16). The actions of estrogens on serum lipids are to reduce serum low-density lipoprotein (LDL) cholesterol concentration and to increase serum high-density lipoprotein (HDL) cholesterol concentration (1), which further provides anti-atherosclerotic effects.

Estrogen influence on the coagulation system

Blood coagulation consists of both pro-coagulation and anti-coagulation processes. Injury of vascular endothelium results in exposure of thrombocytes and activation of coagulation. The anti-coagulation system prevents over-coagulation, a pathological state of thrombosis (thrombophilia). In contrast to males, females show a higher estrogen-related physiological thrombophilia. Estrogens regulate coagulation by decreasing anticoagulant proteins and antifibrinolytic proteins, and increasing platelet adhesiveness (196), which is beneficial fora fetal implantation and a better placental

function during pregnancy (229). Therefore, pregnant women and women on oral contraceptives have higher risk of hypercoagulability.

1.2.4 Estrogen actions on other systems

Estrogen receptors are widely expressed in almost all body tissues and systems, such as bones, brain and immune system, and thus mediate critical widespread non-reproductive effects. The major physiological effects of estrogen on bone are to reduce bone resorption and increase bone formation. Estrogens suppress the synthesis of bone-resorbing cytokines (108), such as interleukin-1, interleukin-6 and tumor necrosis factors, and directly induce apoptosis of osteoclast cells (94). On the other side, estrogens inhibit apoptosis of osteoblast cells and increase proliferation and differentiation of the osteoblast directly (112). In brain, estrogens promote neuronal growth by protecting neurons from oxidative stress and ischemic injury and stimulating production of nerve growth factors (139). Some other estrogen effects on brain include regulating affective state and mood, modulating motor coordination, decreasing seizure threshold and influencing pain pathway (139). In the urinary system, estrogens prevent recurrent urinary tract infection in both young and postmenopausal women by improving urothelial defense mechanisms (132). Lastly, in the immune system, estrogens promote a more robust immune response by stimulating T cells and antigen-presenting cells (101).

1.3 MENOPAUSE

1.3.1 Hormonal and pathophysiological changes during menopause

Menopause is the end of menstruation and fertility, described as the permanent cessation of the primary functions of the ovaries, that is the growth of ovarian follicles and release of female sex hormones: estrogen and progesterone. Three types of circulating estrogens, which are mostly produced by the ovary prior to menopause, will decrease by 60% with major decrease in estradiol (E2) after menopause (25). The average age of menopause in U.S. is 51.3 years old according to the Massachusetts Women's Health Study (5) and the typical age range is between 40 and 61 years old (147). Although the average age for menopause in America is 51, menopausal symptoms can manifest even while a woman is in her forties. If one lives 80 years, then at least one-third or even one-half of a woman's life will be "postmenopausal". During this extended life period she will likely suffer from debilitating physical, mental, and emotional symptoms, including headache, hot flashes, joint and muscle pains, sexual and urinary dysfunction, sleeplessness, and substantial "mood swings" affecting her and those around her.

The perimenopausal period refers to the 2 to 8 years before and 1 year following the final menstrual period defined by World Health Organization (WHO) (228). During menopausal transition, the female body responses to the dramatic dropping of estrogens, and thus appears their regular menstrual cycles as well as many menopausal symptoms. Emotional symptoms includes headache, hot flashes, pains, sexual and urinary dysfunction, sleeplessness, and substantial "mood swings"; other changes includes brittle

nails, hair loss and joint and muscle pains. However, the even more devastating fact is that decreased estrogens increase the risk and incidence of vascular disease, dyslipidemia, and osteoporosis. Low circulating estrogens and low affinity to their receptors increase total cholesterol and triglyceride (136), alter coagulation factors to a more thrombogenic state (140), promote vasoconstriction, decrease endothelium functions, and enhance bone resorption (113). Briefly, there is no doubt that menopausal women are more susceptible to these diseases with age.

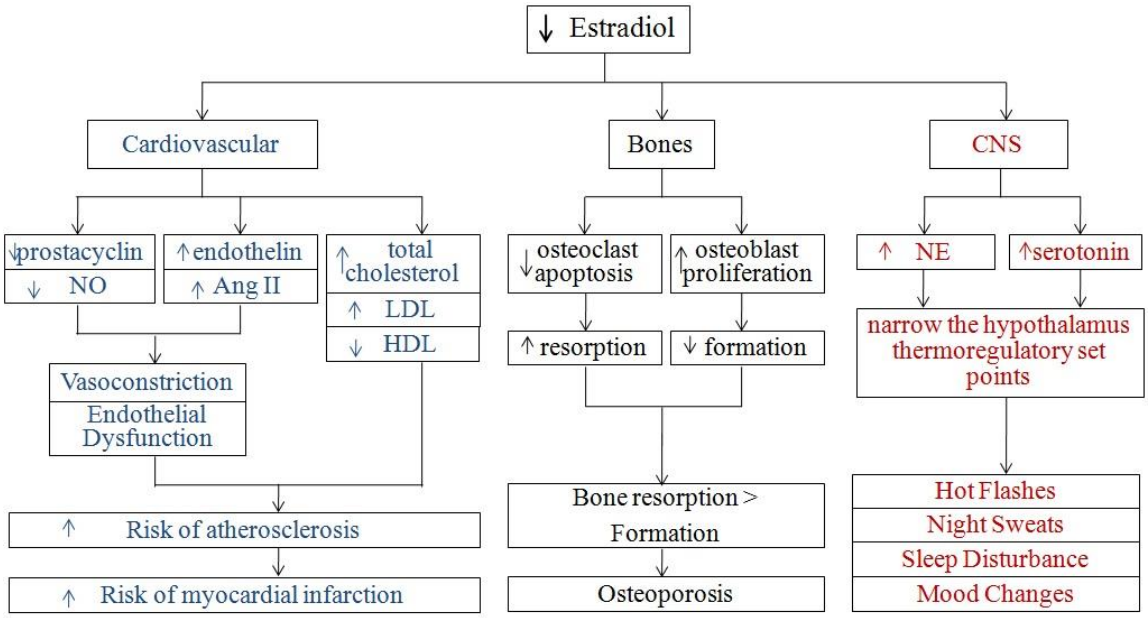

Figure 2. Pathophysiology of menopause changes in cardiovascular system, bones and central nervous system. CNS: central nervous system. NO: nitric oxide. Ang II: angiotensin II. HDL: high density lipoprotein. LDL: low density lipoprotein. NE: norepinephrine. Adapted from Ref. 229.

1.3.2 Prevalence of cardiovascular disease in postmenopausal females

The deadliest consequence of menopause is cardiovascular disease. Premenopausal women aged from 20 to 39 years old have lower incidence of cardiovascular diseases, compared to the same age group of men; the gender difference dramatically decreases with the increase of age and becomes no different during the perimenopausal period (40-59 years old); while postmenopausal females > 60 years old even have higher incidence compared to the same aged males (Fig. 3). Women also show an significant increase in the incidence of coronary heart disease after bilateral oophorectomy (191). Nearly one in three postmenopausal women will die due to complications of cardiovascular diseases, in comparison, however, deaths due to breast cancer comprise only 2.8% (2). Most of cardiovascular diseases is coronary heart disease, also known as atherosclerotic heart disease or coronary artery disease.

Coronary arteries run on the surface of the heart and deliver nutrient and oxygen-rich blood to the distal myocardium. The two major coronary arteries that branch off from the root of the aorta are the left main coronary artery and right coronary artery, after which the left main artery branches into left anterior descending artery (LAD) and circumflex artery. Coronary artery disease is caused by buildup of plaque along the inner walls of coronary arteries, which narrows down the lumen of arteries and reduces blood flow to the distal myocardium. The left anterior descending artery is involved in 45% of atheroma; the right coronary artery is involved in 35%; and the left circumflex coronary artery is involved in 15% (22). Additional to the loss of estrogens' inhibition of the atherosclerosis process, deficiency of estrogen also puts females into high coagulant

states, thus making them at high risk of thrombogenesis, which worsens the condition of atherosclerosis.

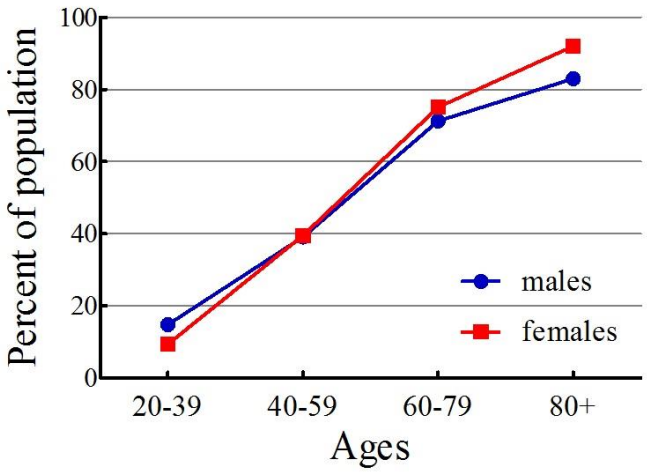

Figure 3. Percentage of population affected by cardiovascular disease between males and females with increasing age. The data is from National Health and Nutrition Examination Survey (NHANES) from 1999 to 2004, including coronary heart disease, heart failure, stroke and hypertension.

1.3.3 Menopausal hormone replacement therapy

Hormone replacement therapy (HRT) has been prescribed as a medication of menopause by physicians over 4 decades. A national survey (103) of postmenopausal women conducted in 1995 in U.S., aged from 50 to 75 years old, reports that 37.6% of women were taking HRT at the survey time. Obviously, HRT has been believed to be beneficial for postmenopausal women.

Undoubtedly, HRT, referring to either estrogen alone or combined with progesterone treatment, is the most effective way to control menopausal symptoms,

particularly vasomotor symptoms and urogenital symptoms. The frequency of vasomotor symptoms are usually ameliorated within 4 weeks after starting treatment and reach maximal effects by 3 months (157). HRT improves vaginal symptoms by decreasing vaginal atrophy and increasing vaginal lubrication with topical oestrogen preparations (67). But all these symptoms seem to recur if stopping treatment. Additionally, HRT is the first line medication for the prevention and management of osteoporosis and bone fracture in menopausal women who are under age of 50 years. Estrogen plus progestin combined HRT reduced the risk of total fracture by 24% and clinical vertebral and hip fracture by one third, compared to placebo (192).

If the higher incidence of coronary heart disease (CHD) in postmenopausal females and females with bilateral oophorectomy is attributed to the lower estrogen level, then replacement of estrogen will decrease and prevent them from the disease. Based on this hypothesis, HRT is recommended to perimenopausal and postmenopausal women as primary or secondary CHD prevention since late 1980s.Supplemental estrogen improves cardiovascular functions by reversing all of these predictable detrimental effects with menopause, through improving high-density lipoprotein cholesterol and lipoproteins (1), inducing coronary and peripheral vasodilation, reducing vascular resistance (63) and improving endothelium functions. Many observational studies (26, 69, 84, 156, 198, 207, 208, 211, 212) have reported a reduction in the risk of coronary artery disease as well as in the mortality from cardiovascular disease and prolong life in postmenopausal women who receive HRT. A meta-analysis of 25 studies

shows approximately 30% decrease in the risk of CHD in HRT users (11), which strongly supports the cardiovascular protective effects of estrogen.

1.3.4 Deleterious effect of HRT

However, the reduction of coronary artery disease in postmenopausal women taking the estrogen therapy might have been caused by some biases which existed in these observational studies (10, 155, 174). Postmenopausal estrogen users usually are well educated and healthier, which offers more protection form heart diseases; they have a better control of risk factors and receive more physician visits for regular follow-up and prescription renewals (10). Therefore, randomized clinical trials may eliminate these biases and give a more accurate estimation of the efficacy and safety of HRT as a prevention of coronary artery disease in postmenopausal women. Unfortunately, more and more randomized clinical trials are rejecting the concept of HRT used in these observational studies.

Early in 1998, Heart and Estrogen/progestin Replacement Study (HERS) research group reported that there is no statistically significant improvement between the HRT group and placebo group in any of the cardiovascular outcomes and other end points including fracture, cancer and total mortality (205). More of the women in the HRT group experienced higher rates of coronary artery events, venous thromboembolic events and gallbladder diseases starting from year 1 (205). In 2002, Women's Health Initiative enrolled more than 100 thousand women in a series of clinical trials and

year	trial	type of prevention	treatment	participant	endpoint	follow-up duration	outcome
2001	EPAT	primary	17β–estradiol+ statin if LDL-C >4.15mmol/L	199 postmenopausal women>45 years with LDL-C >3.37mmol/L	ultrasonogram	2 years	benefit. Slowed the progression of sub-clinical atherosclerosis when it was intiated in early menopause.
2002	EPAT	primary	CEE+MPA	16608 healthy postmenopausal women 50-79 years	Cardiovascular disease	5.2 years	no benefit, posssible harm. Experienced higher incidence for invasive breast cancer, pulmonary embolism and strokes
2007	WISDOM	primary	CEE or CEE+MPA	5694 healthy postmenopausal women 50-69 years	any of the exclusion criteria comfirmed	10 years	beneficial for CHD and osteoporotic fracture, but harmful for breast cancer
1998	HERS	secondary	CEE+MPA	2763 postmenopausal women >55 years with established CHD	CHD	4.1 years	no benefit, early harm. Experienced more CHD events in 1st year.
2000	ERA	secondary	CEE or CEEE+MPA	309 postmenopausal women > 55 years with angiographically verified CHD	angiogram	3.2 years	no benefit
2001	WEST	secondary	17β-estradiol	664 postmenopausal women > 55 years with recently ischemic stroke or transient ischemic attack	stroke	2.8 years	no benefit, early harm. Increased in vascular events in the 1st year
2001	PHOREA	secondary	17β-estradiol + gestodene	321 postmenopausal women > 55 years with atherosclerosis (intima-media thickness > 1 segment of carotid arteries)	ultrasonogram	48 weeks	no benefit
2002	PHASE	secondary	transdermal estradiol or transdermal estrogen and progesterone	255 postmenopausal women > 55 years with angiographically verified coronary heart disease	myocardial infarction or cardiac death	30.8 months	no benefit, early harm. Increase acute coronary events in the first two years
2002	ESPRIT	secondary	17β-estradiol	1017 postmenopausal women age 50-69 years after first myocardial infarction	reinfarction or cardiac death	2 years	no benefit, possible. Experienced more vaginal bleeding therefore poor compliance
2002	wave	secondary	CEE or CEE+MPA	423 women with 15%-75% coronary stenosis	angiogram	2.8 years	no benefit, potential harm. Worsened coronary angiographic change
2002	HERS II	secondary	CEE+MPA	2321 postmenopausal women (93% of the surviver from HERS)	CHD	6.8 years	no benefit, possible harm. Increased the risk of nonfatal ventricular arrhythmia
2003	WELL-HART	secondary	17β-estradiol or 17β-estradiol +MPA	226 postmenopausal women < 75 years with > 1 coronary artery	angiogram	3.3 years	no benefit
2006	EAGAR	secondary	17β-estradiol or 17β-estradiol +MPA	83 postmenopausal women within 6 month of coronary artery bypass surgery	angiogram	42 months	no benefit. Increased disease progression in non-bypassed native coronary arteries, but decreased progression of saphenous vein graft disease

Table 1. Summary of randomized clinical trials of studying the association between HRT and cardiovascular diseases in postmenopausal women. CHD: coronary heart disease. CEE: conjugated equine estrogens. MPA: medroxyprogesterone acetate. EPAT: Estrogen in the Prevention of Atherosclerosis Trial (90). WHI: Women's Health Initiative study (192). WISDOM: Women's international study of long-duration estrogen after menopause (220). HERS: Heart and Estrogen/Progestin Replacement Study (95). ERA: Estrogen Replacement and Atherosclerosis (86). WEST: Women's estrogen for stroke trial (221). PHOREA: postmenopausal hormone replacement against atherosclerosis (3). PHASE: Papworth HRT and Atherosclerosis Survival Enquiry (39). ESPRIT: estrogen in the prevention reinfarction trial (33). WAVE: women's angiographic vitamin and estrogen trial (224).WELL-HART: Women's Estrogen/progestin and Lipid Lowering Hormone and Atherosclerosis Regression Trial (89). EAGAR: Estrogen And Graft Atherosclerosis Research (169).

assessed the major health benefits and risks of HRT (192). The benefits of estrogen alone or combined with progesterone were not considered to be sufficient enough to outweigh the risks, that HRT even increases the risks of heart attacks, strokes, invasive breast cancer and thrombosis (192). Following this, many clinical trials also reported similar observational evidences. Table 1 is a summary of these studies.

1.3.5 Selective Estrogen Receptor Modulators (SERMs) and Selective Estrogen Receptor Down-regulators (SERDs)

Selective estrogen receptor modulators (SERMs) are a class of synthetic non-steroidal compounds exhibit estrogen receptor agonist or antagonist activity depending on the types of tissue. The first generation of SERM, tamoxifen, has been in use since 1966 for breast cancer and has been approved as the drug of choice for the estrogen receptor-positive breast cancer since 2000 (163). The second generation of SERM, raloxifene, was developed as an alternative treatment of postmenopausal osteoporosis and breast cancer (137). The other SERMS, including toremifine, drolocifene, idoifene, lasofoxifene, arzoxifene et al., are the third generation and can be used in postmenopausal women with osteroporosis and/or vasomotor symptoms. Selective estrogen receptor down-regulators (SERD) are a group of SERMs that are pure estrogen antagonists, such as fulvestrant (ICI182,780). Prior to the Women's Health Initiative trial, SERMs were commonly used as a substitute for HRT, but now the use of SERMs has been limited due to significant side effects.

1.3.6 Other medications for postmenopausal females

Anti-lipids - statins

Many studies have reported that an elevated risk of cardiovascular disease is associated with increased level of low-density lipoprotein cholesterol. Thus, statins, HMG-CoA reductase inhibitors which block the production of cholesterol in liver, is the most effective drug for primary (high cholesterol but without CHD) or secondary (early stage of CHD) prevention of CHD. As secondary prevention, long-term use of statins showed 60% reduction in the number of cardiovascular events (including heart attack and sudden cardiac death) and 17% decrease in the risk of stroke (118). Additionally, statins increase bone mineral density and bone formation in postmenopausal women with hypercholesterolemia, and lower 30-40% of the risk of osteoporosis-related fracture (52). Nevertheless, the harmful effects associated with statin use as primary prevention in postmenopausal women may overweigh its benefits. First, Women Health Initiative observes that statins deteriorate glucose tolerance and produce a 48% greater risk of diabetes mellitus (40), which are high risk factors for cardiovascular disease. Secondly, cholesterol synthesis is essential in neuronal repair. Thus, lipophilic statins, which crossed the blood-brain barrier freely, accelerated the decline of cognitive function in patients with Alzheimer disease (193). Lastly, statins only reduce cardiovascular events in men with no history of cardiovascular disease but not in women (175).

Anti-inflammation and anti-platelets medication - aspirin

Inflammation is involved in all the stages of atherosclerosis from initiation throughout the progression via oxidation of low-density lipoprotein (LDL). Oxidized LDL induces the expression of proinflammatory cytokines which induces migration of inflammatory cells such as macrophages, and accumulation of fatty streaks in the vascular walls (123). In addition, platelets accelerate the progress of atherosclerosis by releasing the inflammatory factors and facilitating inflammatory cells migration towards the lesion sites (124). Therefore, aspirin, as an anti-inflammatory mediator and an anti-platelet factor, has been used for primary and/or secondary prevention of cardiovascular disease among women and men. The Women Health Study (WHS) evaluated aspirin as primary prevention of major cardiovascular events in 39876 healthy postmenopausal women $\geq$ 45 age and showed a 9% reduction in myocardial infarction and 24% reduction in ischemic stroke in women with low dose of aspirin (188). Aspirin also prevents certain cancers. For example, aspirin lowers 21% of melanoma among postmenopausal Caucasian women (61). However, there are some severe side effects associated with the use of aspirin. For example, gastrointestinal hemorrhage increases 40% and the hemorrhagic stroke increases 24% in aspirin users (188). Besides, dual anti-platelet therapy with aspirin and clopidogrel (another oral antiplatelet agent) in patients with stable CHD increases maximal platelet reactivity and aggravates the formation of atheroma (19).

High risk disease medications - ACE inhibitors and β-blockers

One of the major factors associated with cardiovascular diseases in postmenopausal women is the increased level of angiotensin receptors, which attributes to systemic vascular constriction and increase of blood pressure. Angiotension-converting enzyme (ACE) inhibitors interfere with the formation of angiotensin, lower blood pressure and reduce the chances of heart attack. β blockers, which block β-adrenergic receptors and the effects of epinephrine, slow down the heart rate and lower blood pressure. Long-term administration of captropril, an ACE inhibitor, reduces more than 30% of the risk of myocardial infarction in hypertensive patients (222). A 23% more reduction of second myocardial infarction after a first heart attack has been shown in patients of long term use of β-blockers (59). Furthermore, ACE inhibitors reverse oxidative stress and endothelial dysfunction in ovariectomized female rats, providing the evidence that ACE inhibitors may be beneficial in postmenopausal women (237). However, besides the common side effects including hypotension and hyperkalemia, both ACE inhibitors and β-blockers may increase the recurrence of breast cancer (62). ACE inhibitors may also raise the risk of bleeding in postmenopausal women by stimulating the release of basal vascular tissue plasminogen activator (tPA) through increased endogenous bradykinin (178). Additionally, in postmenopausal women with coronary heart disease, β blockers increase fragility fractures (204).

Diet supplements

Although diet antioxidant supplements, such as OMEGA-3 fatty acids are the most commonly used means for people to protect their hearts. Until now, no randomized trials have demonstrated their cardiovascular benefits in primary or secondary prevention of cardiovascular events (7).

To summarize, currently there is no ideal medication for primary or secondary prevention of cardiovascular events in postmenopausal women. And it is extremely urgent to find such a new compound that better targets estrogen signaling and be used ina variety of clinical situations, thus provides new strategies not only for treatment of vascular diseases but of a broad range of estrogen-related diseases.

1.4 G-PROTEIN COUPLED ESTROGEN RECEPTOR 1 (GPER)

1.4.1 The discovery of GPER

G protein-coupled estrogen receptor 1 (GPER), formerly known as G protein-coupled receptor 30 (GPR30), is a G protein-coupled seven transmembrane estrogen receptor. It is first identified as a member of G protein coupled receptor (GPCR) and encoded by the *Gper* gene in late 1990s (28, 162).Then, in 1997, Carmeci et al. (28) reported the strong homology of GPR30 in both estrogen receptor-positive breast carcinoma cell line (MCF-7) and estrogen receptor-negative cell line (MDA-MB-231), indicating GPR30 may be involved in the physiological responses of estrogen-target tissues. In 2000, several groups investigated the functional role of GPR30 and demonstrated the possible link between GPR30 and the functional responses to estrogen

(54, 55). However, not until 2005, GPR30 was well accepted as G protein-coupled estrogen receptor 1 (GPER), based on the evidence that GPR30 directly binds estrogen and initiates responses with 10 fold higher binding affinity than that of ERα, provided almost simultaneously by Thomas et al. (216) and Revankar et al.(187).

1.4.2 Subcellular localization of GPER

The subcellular localization of GPER is still controversial. It has been reported that GPER is only localized in the endoplasmic reticulum; however, it is also evidenced that GPER is present in both the endoplasmic reticulum and the plasma membrane. Filardo et al. (53) showed the expression of GPER on the surface of GPER-transfected HEK293 cells by both fluorescence and confocal microscopy and the same finding was made by Funakoshi et al. (60). In contrast, there is a report that shows GPER is present only on the endoplasmic reticulum, therefore, can only be activated by cell-permeable GPER agonist (187).

1.4.3 Ligands of GPER

Estrogen definitely activates G protein-coupled estrogen receptor but with little receptor specificity because it binds to all three estrogen receptors: ERα,ERβ and GPER. Fulvestrant (ICI 182,780), a SERD, as a pure classical antagonist of ERα and ERβ, interestingly exhibits agonistic activity on GPER (181). Tamoxifen (187) and raloxifene (119), the SERMs, also have been reported to be GPER agonists. Furthermore, estrogenic compounds synthesized from plants (phytoestrogens) exhibit activation of

ERα/ERβ as well as GPER (129). In 2006, the first GPER-specific agonist G-1 was identified for its high affinity for GPER with no binding to the classical estrogen receptors, ERα and ERβ (21). Based on current evidences, G-1 is the most selective agonist for studying the functional role of GPER.

1.4.4 GPER actions on regulation of vascular tone

Several studies have reported GPER protein expression in the cardiovascular system, including heart (115), arteries (76), in endothelial cells (91) as well as smooth muscle cells (76).

It has been reported that activation of GPER induces relaxation of both conduit arteries and resistance arteries in different species. The selective GPER agonist, G-1, directly relaxes isolated mesenteric arteries and aortic rings from mREN2 Lew is female rats (125), rat middle cerebral artery (172), porcine coronary arteries (143) and human internal mammary arteries (75), and the vasodilatory effect can be blocked by the GPER selective antagonist G15 (125). The maximal relaxation of porcine coronary arteries induced by G-1 is 40%-50% in vitro. Meyer et al. (143) reports a maximal relaxation of 38 ± 5 % mediated by GPER agonist G-1, which is consistent with the maximal 44.5 ± 3% relaxation of porcine coronary arteries by 3 μM G-1 demonstrated by our previous work (234). It is observed that both nitric oxide dependent and independent pathway are involved in G-1 mediated relaxation porcine coronary artery. We reported that G-1 treatment induces endothelium-denuded porcine coronary artery relaxation and the NO synthase inhibitor L-NAMA has no effect on the response of the artery to G-1 (235).

However, Meyer et al. show reported that L-NAME may completely abolish the dilatory response induced by G-1 (143). Furthermore, infusion of G-1may remarkably lower acute blood pressure in normotensive Sprague-Dawley rats in a dose-dependent manner (75) and chronic systolic blood pressure in ovariectomized hypertensive mRen2.Lewis female rats (127).

However, there is also contradictory vascular effects of G-1 have been reported. Evidence suggests that estrogen potentiates Angiotensin II induced constriction via transactivation of GPER and epidermal growth factor receptor (EGFR) in human coronary microarteries (12). G-1 induces vasoconstriction in basal perfusion pressure and induces vasodilation when the perfusion pressure is elevated by phenylephrine in the isolated perfused rat kidney (114). Besides, my unpublished data also suggest that activation of GPER enhances endothelin-1 induced vasoconstriction effect in female rats aorta. Together, these evidences suggest that activation of GPER may primarily promote vasodilation, but with a component of vasoconstriction effect.

1.4.5 GPER actions on endothelium

Endothelium functions as a selective barrier between the bloodstream and vessel lumen and an important mechanism to maintain vascular homeostasis via release of multiply vasoactive factors (14). Endothelium also regulates vascular homeostasis by inhibiting inflammation, vascular smooth muscle cell proliferation and thrombosis (14). Endothelium dysfunction in many pathogenic states such as aging, hypertension and coronary heart disease is characterized as imbalance between vasodilators and

vasoconstrictors, reduction in NO bioavailability and alteration of anti-inflammatory effects (73, 85), thus serving as a good predictor of cardiovascular disease.

Activation of GPER may promote endothelium function through increasing NO bioavailability and improving anti-inflammatory effects. For example, activation of GPER increases NO synthesis by enhancing phosphorylation of endothelial nitric oxide synthase (eNOS) in diabetic ovariectomized rats (122) and male Wistar rat (58). In addition to stimulating eNOS, GPER also regulates the release of other vasoactive molecules. In GPER-deficient rats, high dose of acetylcholine $\geq$0.1 μM is observed to stimulate the release of endothelium-derived constricting factors (EDCF) and induce stronger contraction comparing to wild type rats (142), suggesting that activation of GPER may attenuate the release of EDCF. Furthermore, it is reported that GPER decreases cell number in mouse brain microvascular endothelial bEnd.3 cells (91) and stimulates aldosterone induced endothelial cell apoptosis in primary cultured rat aortic endothelial cells (72), suggesting an important role of GPER in balancing the endothelial cell proliferation and preventing excessive proliferation. GPER also reduces the inflammatory cytokine tumor necrosis factor (TNF) actions on pro-inflammatory proteins, for example intercellular cell adhesion molecules-1 and vascular cell adhesion molecules-1; this effect is completely abolished by the selective GPER antagonist G-15 (31). These findings suggest that GPER may exert anti-inflammatory and anti-atherogenic effects on endothelium.

1.4.6 GPER actions on vascular smooth muscle

Vascular smooth muscle composes the major part of vessel walls and regulates the redistribution of blood by changing the volume of vessels through contraction/relaxation, as above discussed that activation of GPER relaxes both conduit artery and resistance artery tone; and in the meanwhile contains vasoconstriction component.

Normally, vascular smooth muscle (VSM) cells remain in contractile phenotype with no/little proliferation and express specific contractile markers such as smooth muscle myosin heavy chain and smooth muscle actin (170). When there is vascular injury, VMS cells initiate proliferation cycle and migrate into intima in response to cytokines, such as vascular cell adhesion molecules-1, secreted by damaged endothelial cells (170). The switch of contractile VSM cells to synthetic VSM cells plays a major role in many diseases including atherosclerosis, hypertension and restenosis. Recently, we reported that activation of GPER decreased proliferation of porcine and human coronary artery smooth muscle cells (SMCs) and completely inhibited migration of porcine coronary artery SMCs induced by serum (120). Haas et al. also showed the same observation that GPER reduces proliferation of human umbilical vein VMS cells (75).

In the series studies of the dissertation, we used porcine left anterior descending coronary arteries, as well as porcine and human coronary artery smooth muscle cells to investigate the actions of GPER in coronary arteries and to explore the underlying mechanisms by which GPER contributes to relaxation and constriction.

CHAPTER II

ACTIVATION OF G PROTEIN-COUPLED ESTROGEN RECEPTOR 1 INDUCES ENDOTHELIUM-INDEPENDENT RELAXATION OF CORONARY ARTERY SMOOTH MUSCLE*

2.1 INTRODUCTION

Estrogen induces a rapid, nongenomic relaxation of coronary arteries in vitro (34, 151) and can also enhance blood flow in intact hearts (160, 184). On the other hand, more recent studies indicate that estrogen can contract coronary arteries by increasing oxidative stress under certain conditions (226). Clinical trials indicate that estrogen replacement therapy (ERT) may increase the risk of coronary heart disease in postmenopausal women (192), thus limiting the utility of ERT. As an alternative, selective estrogen receptor modulators (SERMs) are increasingly being employed (197). However, the mechanism(s) of SERM action, especially in cardiovascular tissues, is largely unknown. SERMs and other estrogen-like compounds may function as either agonists, antagonists, or partial agonists of classic estrogen receptors (ERα and ERβ), and increasing evidence indicates that some of these compounds may also activate a more novel transmembrane estrogen-binding protein, G protein-coupled estrogen

*Reprinted with permission from "Activation of G protein-coupled estrogen receptor induces endothelium-independent relaxation of coronary artery smooth muscle" by Yu X, Ma H, Barman SA, Liu AT, Sellers M, Stallone JN, Prossnitz ER, White RE, Han G, 2011. *American Journal of Physiology -Endocrinology and Metabolism,* 301(5), E882-888, Copyright [2011] by American Physiological Society.

receptor 1 (GPER) (55, 181).

Although studies have implicated GPER in mediating effects of estrogen on protein transcription and cell proliferation, the potential role of GPER in mediating nongenomic effects of estrogen in the vasculature remains to be established. Infusion of a selective GPER agonist (G-1) reduced mean arterial pressure acutely in rats and relaxed human mammary arteries in vitro (75); however, a molecular effector mechanism underlying GPER-mediated vasorelaxation was not identified. Moreover, there is virtually nothing known about GPER signaling in coronary arteries. We now provide tissue and cellular evidence that GPER can mediate estrogen signaling in coronary arteries: endothelium-independent relaxation, which involves stimulation of large-conductance, calcium- (BK_{Ca}) and voltage-activated potassium channels expressed in coronary artery smooth muscle (CASM). These findings provide direct evidence that signaling via GPER activation can contribute to the vascular effects of estrogens and suggest a potential rationale for why some SERMs may offer benefits of traditional ERT with fewer potential side effects.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture

Human coronary artery smooth muscle cells (SMCs) were purchased from Cambrex and grown in phenol red free smooth muscle growth medium with 5% fetal bovine serum (FBS), as described previously (227). Steroid hormones and growth factors were removed from FBS by charcoal stripping. MCF-7 cells were purchased

from American Type Culture Collection. Porcine coronary arteries were isolated from fresh porcine hearts obtained from a local abattoir. Single CASM myocytes were isolated as described previously (225). Briefly, the media layer from fresh coronary arteries was dissected free from adventitia and gently shaken (37°C) for 30 min in a dissociation medium containing 45 μM papain, 4 mM dithiothreitol, and 0.2% bovine serum albumin. Cells were then dissociated by gentle trituration. Experiments were performed 6–8 h after isolation.

2.2.2 Tension studies

Porcine coronary arteries were obtained as described previously (226, 227). Briefly, left anterior descending (LAD) coronary arteries were dissected and cleaned of excess fat and connective tissue. Two to four 5-mm rings were obtained from each LAD and prepared for isometric contractile force recordings. To control for possible indirect effects of endothelium-derived vasoactive factors, the endothelium was removed physically by rubbing the intimal surface and tested by observing the absence of acetylcholine-induced relaxation of the initial stabilizing contraction. Rings were mounted on two triangular tissue supports, with one support fixed to a stationary glass rod and the other attached to a force displacement transducer. Isometric contractions or relaxations were recorded on a PC computer using MacLab software. The tissue bathing solution was the modified Krebs-Henseleit buffer (in mM): 122 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.8 $CaCl_2$, and 11.5 glucose, pH 7.2. The solution was oxygenated continuously (95% O_2-5% CO_2) and maintained at 37°C. Coronary artery ring preparations were equilibrated for 90 min under an optimal resting tension of

2 g, and fresh bath solution was added to the tissue chamber every 30 min to prevent accumulation of metabolic end products. After the initial equilibration, preparations were exposed to effective concentrations of a contractile agonist, i.e., 1 μM prostaglandin (PG)F2α, to insure stabilization of the muscles. Vasodilatory responses were calculated as the percent reduction in tension (i.e., relaxation) from the precontracted state. Pharmacological inhibitors were allowed to equilibrate with the arteries for ≥30 min prior to measurement of a complete G-1 concentration-response relationship (1–3,000 nM). One vessel was exposed only to the constrictor agent to control for potential fading of the contractile response. In addition, control responses to DMSO vehicle alone were also measured, and the slight relaxation response to higher concentrations of DMSO was substracted from responses to G-1.

2.2.3 Patch-clamp studies

Cell-attached and excised patch-clamp recordings on isolated coronary myocytes were performed as described previously (225, 227). Whole cell and cell-attached patch-clamp recordings on single cells were performed as described previously (225). For perforated-patch, whole cell recordings, cells were placed in a recording solution of the following composition (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 20 HEPES, and 20 glucose (pH 7.2). Patch pipettes (≤3 MΩ) were fabricated from capillaries by a P-2000 laser pipette puller (Sutter Instruments). The tip of patch pipette was filled with a solution containing (in mM) 90 KCH_3SO_3, 40 KCl, 5 $MgCl_2$, and 20 HEPES to approximate normal cellular [K^+] and [Cl^-] (pH 7.2 with KOH). The remainder of the pipette was back-filled with a similar solution to which 200 μg/ml amphotericin B

(diluted by sonication from a 50 mg/ml stock in dimethylsulfoxide) was added. Voltage clamp and voltage pulse generation was controlled with an Axopatch 200B patch-clamp amplifier (Molecular Devices). Data were acquired and analyzed with pCLAMP 10.0 (Molecular Devices). Voltage-activated currents were filtered at 2 kHz and digitized at 10 kHz. Capacitative and leakage currents were subtracted digitally. For cell-attached patch studies, the recording chamber contained the following solution (in mM): 140 KCl, 10 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, and 30 glucose (pH 7.4, 22–25°C). Patch pipettes (2–5 MΩ) were filled with Ringer's solution (in mM): 110 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES. Voltage across the patch was controlled by clamping the cell at 0 mV with the high-concentration extracellular potassium solution. In experiments recording the potassium channel activity of inside-out patches, the bathing solution exposed to the cytoplasmic surface of the membrane consisted of the following (in mM): 60 K_2SO_4, 30 KCl, 2 $MgCl_2$, 0.16 $CaCl_2$, 1 BAPTA (pCa 7), 10 HEPES, 5 ATP, and 10 glucose (pH 7.4, 22–25°C). The pipette solution was the same Ringer's solution described above. Average channel activity (expressed as number of channels × single-channel open probability; NPo) in patches with multiple BK_{Ca} channels was determined as described previously (225). NPo calculations were based on 10–15 s of continuous recording during periods of stable channel activity. Although channel activity was observed at a variety of membrane potentials, most single-channel data were analyzed at a potential of +40 mV, where BK_{Ca} channel openings are easily distinguished from other channel species to permit more accurate statistical analysis (77).

2.2.4 RT-PCR

Total RNA was extracted from human CASM cells and MCF-7 cells and porcine coronary arteries using the TRI reagent (Sigma-Aldrich). Deoxyribonuclease 1 was used in the extracts to remove any genomic DNA contamination. QiagenOneStep RT-PCR kit was used in this study. The sequences of the primers for GPER were as follows: porcine coronary arteries, sense 5′-GTGGCCGACTCCCTGATCG-3′ and antisense 5′-CGGGCATGGTGCTTGGTGC-3′ (product size is 200 bp) (27); human coronary artery SMCs, sense 5′-GGCTTTGTGGGCAACATA-3′ and antisense 5′-CGGAAAGACTGCTTGCAGG-3′ (product size is 679 bp). PCR was performed for 40 cycles in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany): 94 for 1 min, 55 for 1 min, and 72 for 1 min. β-Actin primers (sense 5′-AGTCCTGTGGCATCCACGAAACTA and antisense ACTGTGTTGGCGTACAGGTCTTTG) were used as internal standard (121). The PCR products were analyzed on 1.5% agarose gel and stained with ethidium bromide.

2.2.5 Western blot

Western blotting analysis was used to detect GPER. After being cleaned of excess fat and connective tissue and having endothelium removed by rubbing the intimal surface, LAD coronary arteries were snap-frozen in liquid nitrogen, pulverized (Fisher Scientific), and then lysed in homogenization buffer of the following composition: 50 mM Tris·HCl, 0.1 mM EGTA, 0.1 mM EDTA, 0.1% SDS, 1% NP-40, and 0.1% deoxycholic acid. Human CASM cells and MCF-7 cells were harvested and homogenized in the same solution. Protein concentrations were determined by Bio-Rad

DC protein assay and separated on SDS-polyacrylamide gels with a Mini Protean II (Bio-Rad) gel kit according to the manufacturer's instructions. Proteins were then transferred to Hybond enhanced chemiluminescence (ECL) membrane (Amersham Pharmacia Biotech) with a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 100 V for 1 h. Membranes were blocked with 5% nonfat milk for 1 h at room temperature and then rinsed with Tris-buffered saline-Tween (TBST) three times for 15 min. Membranes were then probed with GPER primary antibody (Santa Cruz Biotechnology) in TBST containing 5% nonfat milk protein for overnight at 4°C. After washing, the membranes were then incubated with anti-rabbit IgG conjugated to horseradish peroxidase and visualized with an ECL system (Amersham). The membranes were stripped and then immunoblotted with β-actin antibody (Sigma-Aldrich) for protein loading control.

2.2.6 Statistical analysis

Data were expressed as means ± SE. Significance between two groups was evaluated by Student's *t*-test for paired data. Comparison between multiple groups was made by analysis of variance. Probability of <0.05 indicated a significant difference.

2.2.7 Materials

Antibodies were purchased from Santa Cruz Biotechnology, and G-1 was purchased from Calbiochem. All other chemicals were purchased from Sigma-Aldrich. Primers were purchased from Integrated DNA Technologies.

2.3 RESULTS

2.3.1 GPER mediated coronary artery relaxation

GPER is activated by 17β-estradiol and other estrogen-like compounds; however, the cardiovascular effects of these agents are poorly understood. To better target and identify the effects of GPER stimulation in CASM, we measured relaxation of endothelium-denuded porcine coronary arteries in response to G-1, which exhibits nanomolar affinity for GPER but does not bind ERα or ERβ(180). Endothelium-denuded coronary arteries were precontracted with 1 μM PGF2α, and cumulative addition of G-1 produced a concentration-dependent relaxation in coronary arteries. On average, the maximal relaxation effect of G-1 was 44.5 ± 3.2% (3 μM, n = 23; Fig. 4A) with an average EC_{50} of 6.28×10^{-8} M. We then verified the specificity of action on GPER by employing a selective GPER inhibitor, G15 (43). Pretreating arteries with 3 μM G15 significantly attenuated G-1-induced relaxation at all G-1 concentrations above 1 nM (Fig. 4C). The average maximal G-1-induced (3 μM) relaxation was reduced nearly one-half (10.5 ± 2.0%, $n = 21$, $P < 0.003$) by G15 treatment; however, there was no significant change in EC_{50} value (5.2×10^{-8}) in the presence of G15. However, parallel studies indicated a minor relaxation effect in the presence of solvent (DMSO) alone (Fig. 4A, top curve). Care was taken to assure that the total concentration of DMSO never exceeded 0.1% at any time. Therefore, to obtain a more accurate response to G-1, the G-1 concentration-response relationship was normalized by subtracting out this control component, and the resulting G-1 concentration-response relationship is illustrated in Fig. 4B.

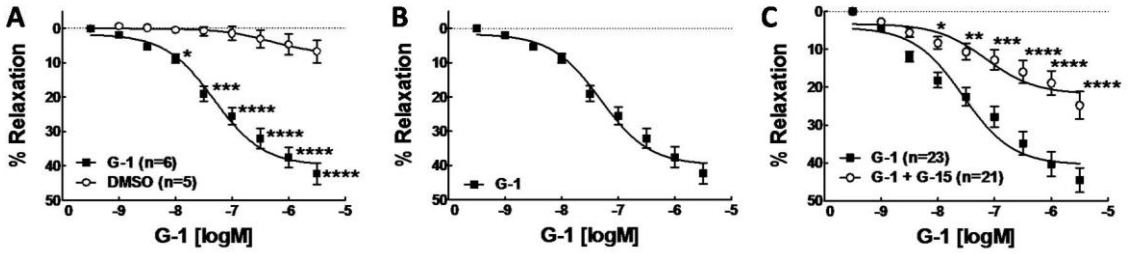

Figure 4. Stimulation of G protein-coupled estrogen receptor 1 (GPER) relaxes endothelium-denuded, precontracted coronary arteries. *A*: complete concentration-response relationship for G-1-induced relaxation. Each point represents the mean relaxation effect ± SE (n = 29). Top curve is a control concentration-response relationship in the presence of solvent (DMSO) alone (n = 6). *B*: actual G-1-induced relaxation response after normalization by subtracting the control component. *C*: complete concentration-response relationship for G-1-induced relaxation in the presence or absence of G15, a GPER antagonist. Each point represents the mean relaxation effect ± SE (n = 21). *P < 0.05.

2.3.2 GPER expression in coronary artery smooth muscle cells

Molecular experiments were done to confirm GPER expression in CASM. RT-PCR studies employed primers for both porcine and human GPER. RT-PCR results demonstrated that high levels of GPR30 mRNA expression were detected successfully in both human and porcine coronary artery SMCs (n=3; Fig.5) together with β-actin (data not shown). These findings are the first to report GPER mRNA expression coronary artery. Previous studies demonstrated that MCF-7 breast cancer tumor cells express GPER (55), and these cells were employed as a positive control for GPER mRNA in each study. In addition to these RT-PCR studies, immunoblotting experiments detected expression of an ~38-kDa GPER protein in CASM from either porcine or human coronary artery SMCs (n=3; Fig.5B). GPER expression in MCF-7 cells was again employed as a positive control.

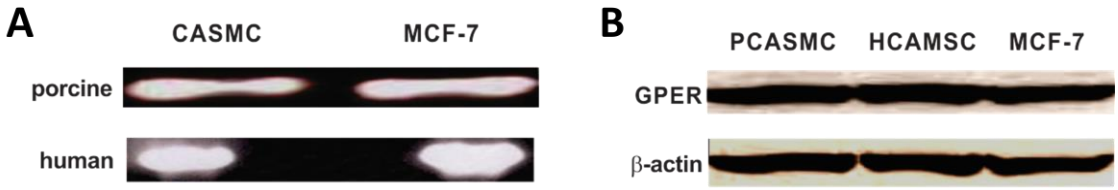

Figure 5. Coronary artery smooth muscle cells (SMCs) express GPER. *A*: GPER mRNA is expressed in porcine and human coronary artery smooth muscle cells (CASMC). RT-PCR products from MCF-7 cells were employed as positive controls. *B*: Immunoblot detection of GPER protein expression in coronary myocytes from either porcine (P) or human (H) arteries (n=3). MCF-7 cells were employed as positive controls. β-actin was employed as a control for protein loading.

2.3.3 BK_{Ca} channel activity involved in NO independent coronary artery relaxation

We had demonstrated previously that estrogen-induced relaxation of CASM (via ERα) involves nitric oxide (NO) production and activation of the large-conductance BK_{Ca} channel (80). In contrast, inhibiting NO signaling had no effect on G-1-induced coronary artery relaxation. Inhibition of NO synthase (NOS) activity with N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 μM) did not attenuate G-1-induced coronary artery relaxation significantly at any concentration ($P>0.05$, n=6; Fig. 6A). In addition, G-1-induced relaxation was unaffected by pretreating arteries with 10 μM[1]H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, a selective inhibitor of guanylyl cyclase activity (n=5; data not shown). However, similar to 17β-estradiol, the relaxation effect of G-1 was dependent upon potassium efflux from CASM cells. G-1-induced coronary artery relaxation was inhibited by 100 nM iberiotoxin, a highly selective inhibitor of BK_{Ca} channels (195). After the data were normalized by controlling for vehicle effects, iberiotoxin inhibited G-1-induced relaxation (1 μM) by an average of ~65% [control 18.7 ± 1.4, iberiotoxin (IBTx) 6.8 ± 1.3%, n=5, P=0.001; Fig.6B]. A completely

normalized concentration-response relationship for G-1-induced relaxation in the absence and presence of 100 nM IBTx is illustrated in Fig. 6C and indicates a significant inhibition of G-1-induced relaxation at all concentrations >10 nM (n=9).

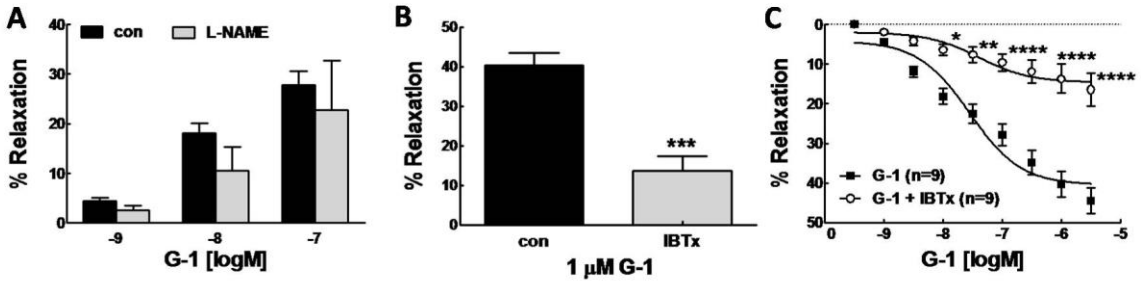

Figure 6. GPER-mediated coronary artery relaxation is nitric oxide independent and involves large-conductance, calcium-activated potassium (BK_{Ca}) channel activity. *A*: pretreating (30 min) coronary arteries with 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) to inhibit nitric oxide synthase had no effect on G-1-induced relaxation. Each bar represents the mean ± SE (n=6). *B*: pretreating vessels (30 min) with 100 nM iberiotoxin (IBTx; 30 min; n=5) attenuated 1 μM G-1-induced relaxation significantly (*P = 0.001). *C*: complete concentration-response relationship for G-1-induced relaxation in the absence or presence of 100 nM IBTx (*n*=5). *P < 0.05 or lower. con, control.

2.3.4 Identification of GPER stimulation on BK_{Ca} channels

To more directly identify a stimulatory effect of G-1 on BK_{Ca} channels, single-channel patch clamp experiments were performed. In cell-attached patches on intact porcine CASM myocytes, we observed that membrane electrical activity was dominated by a large-conductance, outwardly conducting channel that we have identified previously as the BK_{Ca} channel in these same cells (225, 227). As illustrated in Fig. 7A, we observed rather minimal activity of BK_{Ca} channels under control conditions (25–

28°C, +40 mV). In contrast, BK_{Ca} channel activity increased dramatically after cells were treated with 100 nM G-1. On average, G-1 increased BK_{Ca} channel activity from an NPo of 0.006 ± 0.002 to 0.313 ± 0.092 (n=4, P<0.03). A time course plot of BK_{Ca} channel activity is illustrated in Fig.7.We confirmed the identity of this molecule as the BK_{Ca} channel by measuring activity of excised inside-out membrane patches from porcine CASM myocytes (Fig.7B). Under physiological concentrations of calcium (100 nM) at the cytoplasmic surface of the membrane, channel activity was minimal. In contrast, when $[Ca^{2+}]_i$ was raised to 100 μM, there was a dramatic increase in channel opening (Fig. 7B, middle). On average, channel NPo was increased from 0.001 ± 0.001 to 0.684 ± 0.178 (n=3, P<0.05) by increasing $[Ca^{2+}]_i$. Furthermore, subsequent addition of 1 mM tetraethylammonium (TEA) abolished calcium-stimulated channel activity (Fig. 7B, right). At concentrations of 1 mM or less, TEA exhibits selectivity for blockade of BK_{Ca} channels.

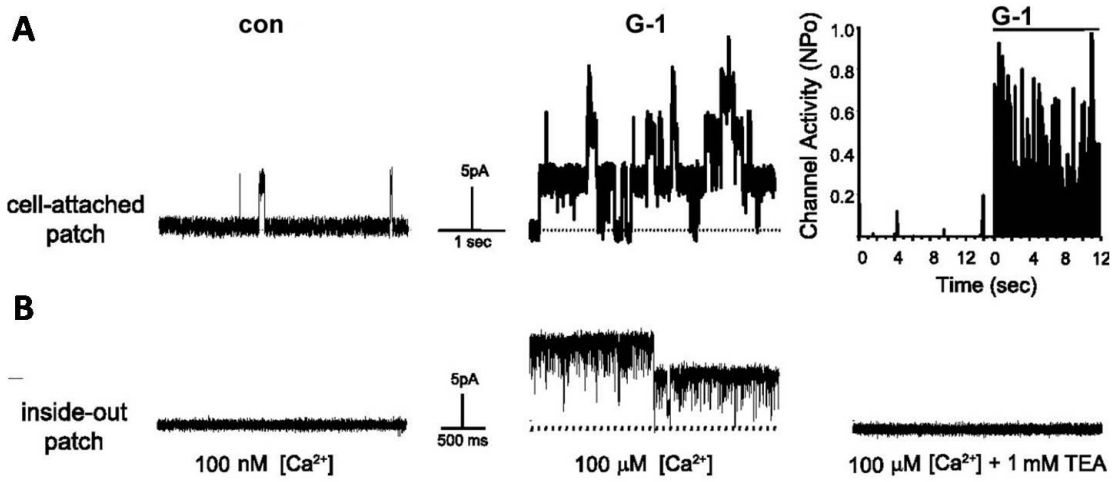

Figure 7. G-1 stimulates BK_{Ca} channel activity in porcine coronary artery SMCs. *A*: typical recordings from the same cell-attached patch (+40 mV) before (con) and 20 minutes after application of 100 nM G-1. Upward deflections indicate channel opening from the closed state (dashed line). Right: activity histogram of BK_{Ca} channel open probability (NPo) before and 20 minutes after application of 100 nM G-1. Total recording time under each condition was 12–14 s. Break in the time axis represents drug incubation. *B*: typical recordings from the same excised inside-out patch from a porcine coronary artery SMCs with either 100 nM (left) or 100 μM (middle) [Ca^{2+}] exposed to the cytoplasmic face of the membrane (+40 mV). Right: recording after subsequent addition of 1 m TEA to the cytoplasmic surface of the membrane (100 μM [Ca^{2+}]).

We further confirmed the ability of G-1 to open a BK_{Ca} channel in CASM by observing a stimulatory effect of G-1 in myocytes from human coronary arteries (human CASM). As illustrated in Fig. 8A, addition of 100 nM G-1 to a cell-attached patch on a human CASM cell increased channel activity substantially. On average, channel activity increased from an NPo of 0.012 ± 0.004 to 0.027 ± 0.003 (n=3, P=0.05; Fig. 8, right). In contrast to these experiments on intact myocytes, G-1 was unable to stimulate BK_{Ca} channels isolated in a cell-free, inside-out patch (Fig.8B). When we excised the membrane patch into the microscope chamber, the cytoplasmic surface of the patch was immediately exposed to a supra physiological level of calcium (100 μM). As expected,

this resulted in a dramatic increase channel activity, confirming that this protein is indeed the BK_{Ca} channel that we have characterized extensively in these same cells previously (227, 240). When [Ca^{2+}] was subsequently lowered to a more physiological 100 nM, channel activity immediately decreased as expected. However, in contrast to what we observed in cell-attached patches, addition of 100 nM G-1 to this excised patch had no effect on BK_{Ca} channel activity (Fig. 8B, right). These experiments provide direct evidence that excludes potential effects of G-1 directly on the BK_{Ca} channel protein and demonstrate the necessity of an intact cellular transduction mechanism.

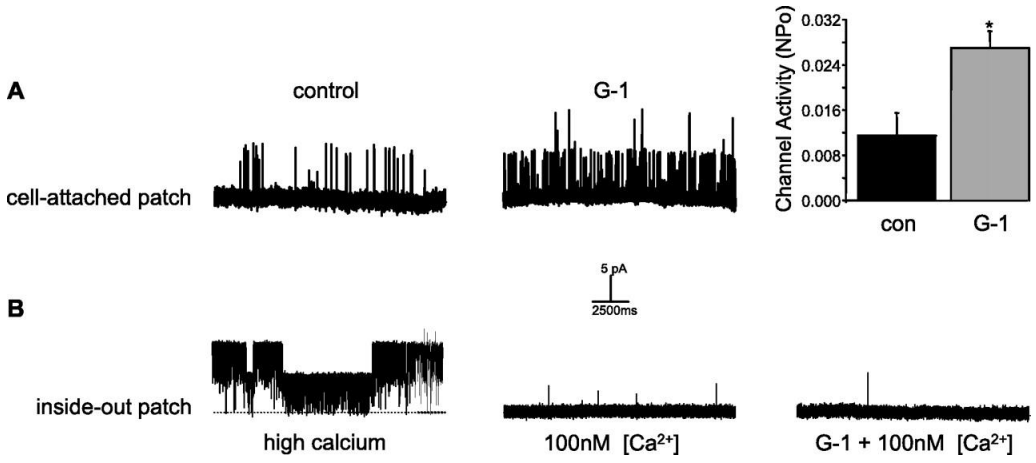

Figure 8. G-1 stimulates BK_{Ca} channel activity in human coronary artery SMCs. *A*: recordings (+40 mV) from the same cell-attached patch on human coronary artery SMCs before (control) and 25 min after addition of 100 nM G-1. Upward deflections indicate channel opening from the closed state (dashed line). Right: average increase in BK_{Ca} channel activity (NPo) stimulated by G-1. Bars represent the average of 3 membrane patches ± SE. *B*: recordings from the same excised inside-out patch from human coronary artery SMCs. Upward deflections indicate channel opening from the closed state (dashed line). Left: BK_{Ca} channel activity in the presence of high calcium (100 μM) at the cytoplasmic surface of the patch. Middle: channel activity recorded after calcium levels were lowered to 100 nM. Right: G-1 (100 nM) had no effect on channel activity in excised patch. *$P < 0.05$.

2.4 DISCUSSION

Recent studies suggest that GPER, a nonclassic, membrane-associated, estrogen-binding protein, may mediate rapid estrogen signaling in certain cell types (181); however, the role of GPER in mediating estrogen responses is controversial. For example, GPER-deficient mice exhibit a normal reproductive phenotype, indicating a nonessential role for GPER in mediating hypothalamic-pituitary-gonadal function (167). In contrast, other studies indicate an important if not essential role for GPER in reproductive function. Inhibition of GPER mRNA and protein expression markedly suppresses estrogen-stimulated follicle formation in hamster ovary (223), and estrogen-stimulated proliferation of mouse sperm cells is mediated via GPER (199). Thus, the physiological role of GPER remains unclear, and even less is known of a potential role for GPER in cardiovascular function. Although GPER mRNA has been detected in human blood vessels (76), we know little of its function and cellular/molecular transduction mechanisms in the cardiovascular system.

G-1 is reported to be a GPER agonist that exhibits nanomolar affinity for GPER but does not bind ERα or ERβ (180). We had demonstrated previously that ICI 182,780, a nonselective ER antagonist, relaxes porcine coronary arteries in vitro by an endothelium-independent mechanism (79). Meyer et al. (143) have also reported that G-1 and ICI 182,780 relaxed porcine epicardial coronary artery rings, but the mechanism of G-1 action was not investigated in detail in this study. The present results now demonstrate that G-1 induces endothelium-independent relaxation of coronary arteries and that this response is attenuated by G15 (selective GPER antagonist). These studies

strongly suggest that activation of GPER in CASM causes relaxation. Furthermore, our functional studies are supported by RT-PCR and immunoblot experiments verifying expression of GPER in both porcine and human CASM. Taken together, these findings implicate a role for GPER in mediating acute estrogen and/or SERM action on CASM and hence, cardiac blood flow. Support for this mechanism is gained from prior studies indicating GPER mRNA expression in smooth muscle from human internal mammary arteries and saphenous veins (76) and findings that G-1 administration lowers blood pressure in vivo (75). Therefore, accumulating evidence indicates a novel, nongenomic mechanism mediating the effect of estrogens on coronary arteries, i.e., signaling via a G protein-coupled receptor that does not involve activation of either ERα or ERβ. It is unknown whether our findings on GPER signaling in CASM can be extended to coronary endothelium, which is also a target tissue of estrogen action.

Signaling mechanisms downstream to GPER activation in CASM remain to be elucidated. We have reported that estrogen-induced coronary artery relaxation involves generation of NO in both porcine and human CASM (42, 225, 227). In contrast, we found that inhibiting NOS activity in coronary arteries with L-NAME had no effect on G-1-induced relaxation. Moreover, inhibiting the activity of guanylyl cyclase (a primary target for NO signaling) also had no effect on responses to G-1. Taken together, these findings indicate a NO-independent mechanism of action for G-1/GPER in CASM. These findings are consistent with our previous report that ICI 182,780 does not stimulate NO production in CASM (80). Therefore, we conclude that GPER-mediated relaxation of CASM, unlike 17β-estradiol-stimulated coronary artery relaxation via ERα,

is not dependent upon NO production. The fact that GPER-induced relaxation was endothelium independent further supports the idea that NO was not involved in this response. However, similar to 17β-estradiol, we found evidence that GPER activation leads to stimulation of BK_{Ca} channel activity in CASM.

Our tension studies demonstrated that coronary artery relaxation induced by G-1 involves potassium efflux. These findings implicate potassium channels in the vascular response to GPER activation. The fact that G-1-mediated relaxation was inhibited by iberiotoxin, which is a highly selective antagonist of BK_{Ca} channels, provides strong pharmacological evidence for involvement of this protein in GPER-mediated relaxation. Definitive evidence that GPER activation stimulates BK_{Ca} channel activity in CASM was then provided by single-cell, patch-clamp recordings on myocytes isolated from porcine and human coronary arteries. As we have reported in a number of previous studies (42, 79, 225), ion channel activity in CASM is dominated by a large-conductance (~200 pS) potassium channel that is stimulated by cytoplasmic calcium and inhibited by iberiotoxin. This BK_{Ca} channel is a known target of estrogen action and is an important effector molecule that induces membrane repolarization and CASM relaxation.

We found G-1 to be a powerful stimulator of BK_{Ca} channel activity in intact CASM myocytes from either porcine or human arteries. Interestingly, however, G-1 was ineffective at stimulating channels isolated in excised membrane patches. These findings provide direct evidence that the BK_{Ca} channel protein complex is not a functional substrate for G-1 binding. Rather, G-1 action on CASM requires an intact cellular signaling mechanism involving diffusible cytosolic and/or membrane-associated second

messenger compounds. Although we can find no previous patch-clamp studies that have employed G-1, there is a previous report that ICI 182,780 can stimulate BK_{Ca} channels in colonic smooth muscle cells (47). Therefore, we speculate that the effect of ICI 182,780 in these nonvascular smooth muscle cells is also mediated via GPER. In light of these findings, it is becoming apparent that estrogen and estrogen-like compounds may stimulate distinct receptor proteins in CASM (e.g., ERα, ERβ, and/or GPER), but ultimately these transduction pathways appear to converge on a common effector protein, the BK_{Ca} channel. Thus, the present findings from intact arteries and isolated myocytes are entirely consistent with a novel, nongenomic mechanism of estrogen action in the cardiovascular system, i.e., activation of GPER, which relaxes CASM via stimulation of BK_{Ca} channel activity. We certainly have not excluded other potential signaling mechanisms for GPER but propose BK_{Ca} channel activity to be a likely downstream effector mediating GPER-induced coronary artery relaxation.

In conclusion, because of increased cardiovascular risk associated with ERT, other estrogen-like compounds (e.g., SERMs) have been recommended as an alternative therapy (148). These compounds have a diversity of chemical structure and may have a unique and unpredictable array of clinical responses (197). The present study has demonstrated that, like estrogen, a nonsteroidal GPER agonist (G-1) relaxes endothelium-denuded coronary arteries. These studies indicate a novel, nongenomic estrogen-signaling mechanism in CASM, which we found to express GPER mRNA and protein. More conclusive proof of GPER involvement in these responses will require use of GPER-knockout mice, and future studies will certainly include such additional

measures. Nonetheless, the present studies suggest a common signaling mechanism for nongenomic estrogen action in CASM, i.e., stimulation of GPER leading ultimately to increased activity of BK_{Ca} channels and coronary artery relaxation. Unlike 17β-estradiol, this response does not appear to involve NO production, nor does it require activation of classic ERs. We propose that GPER activation can enhance coronary blood flow and thus may help protect against myocardial ischemia. This hypothesis is supported by a recent finding that G-1 is cardioprotective when administered to rat hearts in vitro (45), although this protection also involved GPER expressed in cardiac myocytes. In summary, these findings strongly support the caveat that estrogen-like compounds used for treating specific disease states (e.g., cancer) may also affect other organs and systems.

CHAPTER III

G PROTEIN-COUPLED ESTROGEN RECEPTOR 1 MEDIATES RELAXATION OF CORONARY ARTERIES VIA CAMP/PKA-DEPENDENT ACTIVATION OF MLCP*

3.1 INTRODUCTION

Cardiovascular disease, especially coronary heart disease, is the leading cause of death among women in the US and exceeds breast cancer mortality in women of all ages (2, 230). Although premenopausal women have had less incidence of cardiovascular disease than men, by the late 1970s the risk of cardiovascular disease had equalized (214). Animal and observational studies have suggested female ovarian sex hormones to protect against cardiovascular dysfunction (168). For example, estrogen alone or in combination with a progestin has favorable effects on lipoproteins and fibrinogen (1), improves vascular inflammation (231), and restores endothelial function and nitric oxide release, thus promoting vasorelaxation (111). Estrogen can also act directly on smooth muscle cells to relax arteries (79, 80) and inhibit smooth muscle cell proliferation (217). Although this evidence strongly suggests that estrogen is indeed vasoprotective, randomized, controlled large trials of hormone replacement therapy such as the Women's

*Reprinted with permission from "G protein-coupled estrogen receptor 1 mediates relaxation of coronary arteries via cAMP/PKA-dependent activation of MLCP" by Yu X, Li F, Klussmann E, Stallone JN, Han G, 2014. *American Journal of Physiology-Endocrinology and Metabolism*, 307(4), E398-407, Copyright [2014] by American Physiological Society.

Health Initiative showed no cardiovascular benefit for estrogen or estrogen plus progestogen treatment (92, 134). These apparent conflicting findings warrant further mechanistic study of the effects of estrogen on the vasculature.

The newly reported, membrane-associated G protein-coupled estrogen receptor 1 (GPER; initially called GPR30) is structurally unrelated to nuclear ERα or ERβ (165, 173, 187, 216). Membrane localization of GPER provides a novel mechanism for rapid, nongenomic responses to estrogen (82, 187). Evidence that GPER plays an important role in mediating important cardiovascular actions of estrogen is building (78, 126, 146, 158). GPER activation relaxes rat carotid (24), rat aorta (127), human mammary arteries (75), and coronary arteries (143, 235) with or without endothelium, indicating that GPER acts directly on vascular smooth muscle to regulate vascular tone. The selective GPER agonist G-1 lowers blood pressure in either normotensive (75) or mRen2.Lewis hypertensive rats (127). Conversely, deletion of *Gper* increases blood pressure (135). G-1 improves functional recovery from myocardial ischemia-reperfusion by reducing post-ischemic contractile dysfunction and infarct size, reduces brain infarct size, and prevents stroke-induced immunosuppression in rats (45, 238). However, because of its novelty, the mechanism by which GPER mediates estrogen action is still unclear (117).

Estrogen stimulates cAMP production in breast cancer cells via activation of GPER (216). Various phyto- and xenoestrogens also stimulate cAMP production via GPER either in breast cancer cells or in an ERα- and ERβ-negative cell line [human embryonic kidney (HEK)-293 cells] that was stably transfected with GPER (215, 216). Although estrogen-stimulated production of cAMP, a powerful vasodilatory signal, has

been demonstrated in rat aortic (48) and porcine coronary artery smooth muscle cells (SMCs) (105), the role of cAMP in response to GPER activation in coronary arteries is unknown. The key component of generating and sustaining vascular tone is the phosphorylation state of the smooth muscle contractile apparatus myosin light chain (MLC) (107). It has been reported that cAMP decreases MLC phosphorylation by activating the MLC phosphatase (MLCP) (4, 6); however, to the best of our knowledge, a potential link between estrogen, cAMP, and MLC has not been investigated. In this study described here, we tested the hypothesis that GPER activation induces coronary relaxation by activating MLCP via cAMP/PKA signaling.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture

Coronary arteries were dissected from porcine hearts obtained from a local abattoir, as described previously (226, 227), and the protocol was in compliance with the Texas A&M University Institutional Animal Use Protocol. The endothelial layer was gently removed with a cotton-tipped swab, and the media layer was dissected free from the adventitia. The medial layer was then enzymatically dispersed in a 25-cm^2 tissue culture flask and gently shaken (37°C) in 10 ml of dissociation medium, containing (in mM) 110 NaCl, 5 KCl, 2 $MgCl_2$, 0.16 $CaCl_2$, 10 HEPES, 10 $NaHCO_3$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 0.48 EDTA, 10 taurine, and 10 glucose plus 24 mg of elastase, 6 mg of collagenase, and 15% bovine serum albumin for 3 h. Cells were then dispersed by gentle trituration and centrifuged at 800 rpm for 5 min. The pellet was suspended with smooth

muscle cells medium SmGM (Lonza) and seeded in a 25-cm^2 tissue culture flask coated with 1% gelatin. Human coronary artery smooth muscle cells (SMCs) were purchased from Life Technologies. Both human and porcine coronary artery SMCs were maintained in SmGM and kept at 37°C under 5% CO_2 in a humidified incubator. The purity of coronary artery SMCs in culture was verified by positive staining with smooth muscle-specific α-actin (120). Passages 2 and 3 of porcine and passage 5 of human coronary artery SMCs were used in this study.

3.2.2 Tension studies

Left anterior descending coronary arteries were dissected from porcine hearts, trimmed of fat and connective tissue, and cut into rings (axial length ~5 mm) for isometric contractile force recordings. Rings were endothelium denuded to eliminate effects of endothelium-derived vasoactive factors. Endothelium denudation was confirmed by the absence of relaxation to bradykinin (100 nM) exposure. Arterial rings were mounted in isometric myographs (Danish Myograph Technology) filled with 10-ml modified Krebs-Henseleit buffer (in mM): 122 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.8 $CaCl_2$, and 11.5 glucose, pH 7.2, bubbled with 95% O_2-5% CO_2 (pH 7.4) at 37°C. Two stainless-steel wires were guided through the lumen of the rings. One wire was connected to a force displacement transducer and the other to a stationary micrometer. The preparations were then allowed to equilibrate for 90 min in Krebs-Henseleit buffer. During the first 30 min, the arterial rings were gradually stretched until a stable tension of 20 mN was obtained, which was the optimal resting tension determined in preliminary experiments for these preparations. Isometric tension

was recorded on a PC computer using the LabChart data acquisition system (AD Instruments). After the initial equilibration, preparations were contracted three times with prostaglandin F2α (PGF2α; 1 μM) and then washed and allowed to relax to basal tension. Then preparations were exposed to PGF2α (1 μM) to induce a stable contraction. When the force reached steady state, G-1 was added in a cumulative manner by increasing the concentration in log increments. Relaxation responses were calculated as the percent reduction in tension at each G-1 concentration from the precontracted state. Pharmacological inhibitors were allowed to equilibrate with the arteries for ≥30 min prior to measurement of a complete G-1 or other vasodilators' concentration-response relationships. One ring in each set of experiments was exposed only to the constrictor agent PGF2α (1 μM) to control for potential fading of the contractile response. In addition, control responses to cumulative amounts of vehicle [dimethylsulfoxide (DMSO)] were measured by adding the identical amount of DMSO used for each concentration of G-1, forskolin, or 6-Bnz-cAMP. The total amount of vehicle never exceeded 0.1%.

3.2.3 Western blot

Standard Western blot analysis was used for detecting phosphorylation of myosin-targeting subunit protein-1 (p-MYPT-1), the regulatory subunit of MLCP, from porcine coronary artery tissue lysates and phosphorylation of MLC from porcine coronary artery SMC lysates. Arterial rings were prepared, mounted in the chamber of the myograph, equilibrated, and contracted three times by PGF2α (1 μM), as described above. Then preparations were pretreated with inhibitors and G-1 30 min before PGF2α

(1 μM) was added to induce contraction. Artery rings were collected when contraction reached plateau and were snap-frozen in liquid nitrogen. Tissues were pulverized (Fisher Scientific) and then lysed in homogenization buffer of the following composition: 50 mM Tris·HCl, 0.1 mM EGTA, 0.1 mM EDTA, 0.1% SDS, 1% NP-40, and 0.1% deoxycholic acid. For MLC20 phosphorylation detection, porcine coronary artery SMCs were treated with drugs, as indicated in the results. Coronary artery SMCs were harvested and homogenized. Protein concentrations were determined by a detergent-compatible protein assay (Bio-Rad). Proteins were separated by SDS-PAGE with a Mini Protean II (Bio-Rad) gel kit according to the manufacturer's instructions.

Proteins were then transferred to Hybond enhanced chemiluminescence membrane (Amersham Pharmacia Biotech) with a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 100 V for 1 h. Membranes were blocked with 5% nonfat milk for 1 h at room temperature and then rinsed with Tris-buffered saline-Tween (TBST) three times for 15 min. Membranes were then probed with primary antibodies in TBST containing 5% nonfat milk protein for overnight at 4°C. The p-MYPT-1 was probed with Thr^{853} p-MYPT-1 primary antibody (Santa Cruz Biotechnology), p-MLC was probed with primary antibody p-MLC (Ser^{19}; Cell Signaling Technology), and β-actin (Santa Cruz Biotechnology) was used for protein loading control in the densitometry analysis. Experiments were performed in triplicate, and means ± SE were calculated and plotted.

3.2.4 Cyclic AMP assay

Cyclic AMP production was measured with a cAMP enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemical), as described

previously (240). Porcine coronary artery SMCs, passages 2–3, were cultured in 35-mm culture dishes in SmGM medium for 2 or 3 days. When cells reached 85–90% confluence, cells were serum-deprived for 18 h in phenol red-free α-MEM medium (Invitrogen). Cells were preincubated for 30 min at 37°C in buffer containing 3-isobutyl-1-methylxanthine (100 μM) to inhibit phosphodiesterases. Then cells were treated with G-1 (10 nM to 1 μM), 0.1% DMSO was used as solvent control, and forskolin (1 μM) treatment was used as a positive control.

3.2.5 PKA activity assay

PKA activity was measured by applying the PepTag protein kinase assay (Promega) according to the manufacturer's instructions (20). Porcine coronary artery SMCs (passage 2) were cultured in 100-mm culture dishes in SmGM medium for 2 days. After being serum-deprived for 18 h, cells were treated with G-1 (100 nM) in the presence or absence of G-36 (1 μM) for 30 min; 0.1% DMSO was used as solvent control, forskolin (1 μM) was used as a stimulant to mediate PKA activation, and GPER agonist and antagonist were applied as described above. Cells were washed with phosphate-buffered saline and suspended in PKA extraction buffer provided in the assay kit. The crude extracts were assayed on the same day that they were prepared to retain maximal activity. The PKA-specific peptide substrate L-R-R-A-S-L-G (Kemptide) was used for measuring PKA activity. The phosphorylated Kemptide was separated from nonphosphorylated Kemptide by 0.8% agarose electrophoresis. The peptides were imaged under UV light. The image was scanned and quantified by densitometry using ImageJ.

3.2.6 RhoA activity assay and phsophorylation of RhoA detection

RhoA activity was monitored by measuring changes in RhoA-GTP in cultured human and porcine coronary artery SMCs under drug treatments. Briefly, human (passage 5) and porcine (passage 3) coronary artery SMCs were seeded in 60-mm dishes. After 2 days of being grown in the SmGM medium, cells were serum-deprived for 18 h and then treated with drugs as indicated in the results. Cell lysates were collected and protein concentrations from each sample quantified and adjusted for RhoA activity assay and Western blotting. RhoA activity was measured with G-LISA RhoA activation assay biochem kit (Cytoskeleton) according to the manufacturer's instructions by detecting absorbance at 490 nm. RhoA phosphorylated at Ser^{188} by PKA was detected by Western blotting using specific antibodies.

3.2.7 Materials

Antibodies were purchased from Santa Cruz Biotechnology. G-1 was purchased from Calbiochem, and 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphorothioate Rp-isomer (Rp-8-CPT-cAMPS) and N6-benzoyladenosine-3′,5′-cyclic monophosphate (6-Bnz-cAMP) were from Biolog Life Science Institute. PKA inhibitor PKI (14-22) amide (myristoylated) is from Enzo Life Sciences. 3,3′-Diamino-4,4′-dihydroxydiphenylmethane, 4,4′-methylenebis(2-aminophenol), and bis(3-amino-4-hydroxyphenyl)methane (FMP-API-1) were obtained as described by Christian et al. (37). All other chemicals were purchased from Sigma-Aldrich.

3.2.8 Statistical analysis

Data are expressed as means ± SE. Significance between two groups was evaluated by Student's *t*-test for paired data. Comparison between multiple groups was made by two-way ANOVA. $P < 0.05$ indicated a significant difference.

3.3 RESULTS

3.3.1 Cyclic AMP production and coronary relaxation

We and others have reported that GPER activation induces a rapid relaxation of porcine coronary arteries (79, 143, 235). However, the downstream signaling events in this pathway have yet to be elucidated. GPER activation has been shown to stimulate cAMP production in breast cancer cells (216). We tested whether activating GPER with G-1 (10–1,000 nM) stimulated cAMP production in porcine coronary artery SMCs. G-1 at every concentration significantly increased cAMP production compared with the DMSO solvent control group ($P < 0.05$; Table 2). The stimulation effect was displayed in a concentration-dependent fashion, which closely paralleled the relaxation effect of G-1 (Fig. 9A). Cumulative addition of G-1 relaxed PGF2α-precontracted (1 μM) endothelium-denuded porcine coronary artery rings (Fig. 9A and B) and a solvent (DSMO) control were performed, and the weakening of the tension with time was not significant (Table 3), as we reported previously (235). The maximal relaxation effect of G-1 was 45.2 ± 8.9%, with an average EC_{50} value of 2.67×10^{-8} M. These data suggest that cAMP contributes to GPER action in porcine coronary artery reactivity.

compound	cAMP content (%)
control	100
G-1 10 nM	230.69 ± 13.32**
G-1 100 nM	291.30 ± 20.83***
G-1 1000 nM	304.71 ± 11.21***
forskolin 1 μM	539.31 ± 148.19

Table 2. G-1 stimulates cAMP production in porcine coronary artery SMCs. Results are standardized by control group. Values are given as mean + SE, n=4. **P<0.01, ***P<0.001, significant difference compared with control by using two-way ANOVA.

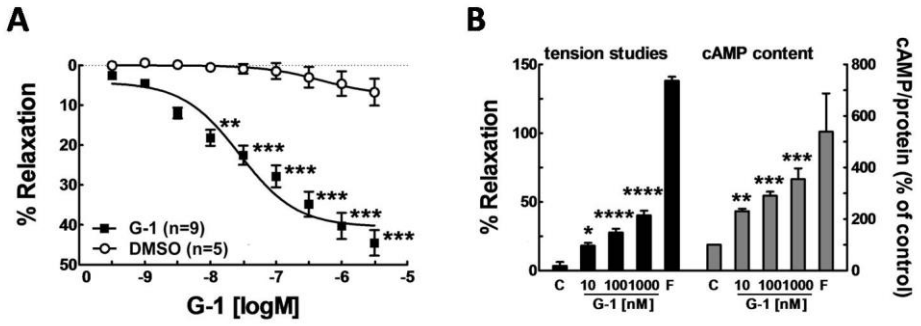

Figure 9. G-1 induces relaxation of precontracted coronary arteries and stimulates cAMP production.*A*: concentration-response relationship for G-1-induced, endothelium-denuded, prostaglandin F2α (PGF2α)-precontracted (1 μM) coronary artery relaxation. Each point represents the mean relaxation effect ± SE. *B*: cAMP production of the porcine coronary artery smooth muscle cells (SMCs) in response to G-1. Cells were treated for 30 min in serum and phenol red-free DMEM in the presence of 3-isobutyl-1-methylxanthine (100 μM) with 0.1% DMSO + G-1 (10–1,000 nM; for each treatment, n = 4) and forskolin (F; 1 μM; n = 4). Each point represents the mean production ± SE. *P < 0.05, **P< 0.01, ***P < 0.001, and ****P < 0.0001 compared with solvent control (C) group. Results are normalized by total protein amount.

Compounds	% reduction of the G-1 relaxation	% relaxation	EC_{50} (μM)
G-1^{+}		44.51 ± 3.21 (n=19)	0.027
SQ-22536 100 μM	68.93%	13.83 ± 3.75 (n=4) **	0.277
Rp-8-CPT-cAMPS 5 μM	33.36%	29.66 ± 2.74 (n=4) **	0.215
PKI 5 μM	57.09%	19.10 ± 2.91 (n=4) **	0.278
FMP-API-1 100 μM	48.80%	22.79 ± 4.20 (n=5)**	0.344
DMSO^{++}		6.72 ± 7.52 (n=5) ****	0.486
Forskolin 3 μM^{+++}		160.26 ± 12.34 (n=6) ****	0.101
6-Bnz-cAMP 3 μM^{+++}		96.31 ± 3.58 (n=4) ***	145.3

Table 3. Effects of* cAMP/PKA signaling *compounds on porcine coronary artery relaxation response to 3 μM G-1. Values are given as mean + SE. The number of experiments is indicated in parentheses. **P<0.01, ***P<0.001, ****P<0.0001, significant difference compared with control by using two-way ANOVA. In the upper rows (row 1 to row 5), artery rings were pretreated with each of the inhibitors and the results were compared to G-1 alone group ($^{+}$G-1). In the lower rows (row 6 to row 8), $^{++}$DMSO group was used as solvent control. $^{+++}$Forskolin and 6-Bnz-cAMP were used as vasorelaxant agents and the results were compared to DMSO group.

3.3.2 The functional role of cAMP

Next, we tested the functional role of cAMP in GPER-mediated coronary artery relaxation. First, the specific adenlylyl cyclase (AC) inhibitor SQ-22536 was used to inhibit cAMP production in these artery rings. As shown in Fig. 2A, SQ-22536 (100 μM) markedly decreased G-1-induced relaxation, reaching statistical significance at 300 nM to 30 μM, increasing EC_{50} of G-1 from 27 to 277 nM (Table 3). Then the AC activator forskolin was used to increase cAMP production in these preparations. As expected, forskolin generated a strong concentration-dependent relaxation of endothelium-denuded coronary artery rings. The relaxation response to forskolin reached statistical significance at concentrations as low as 30 nM, and the EC_{50} was 101 nM (Table 3 and Fig. 10B). The maximal effect of 30 μM forskolin was a 160.3 ± 21.4% reduction in precontracted tension, also revealing a significant level of resting tone in these rings.

These data suggest that cAMP is a powerful vasorelaxant in porcine coronary artery and is involved in the GPER-mediated coronary relaxation.

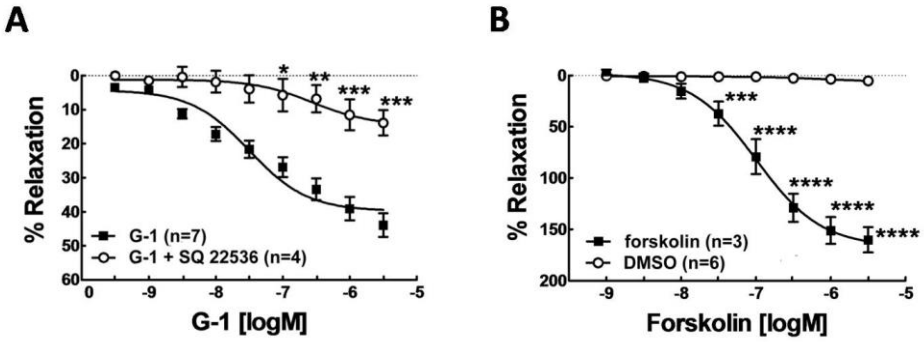

Figure 10. cAMP is involved in G-1-induced porcine coronary artery relaxation. *A*: concentration-response relationship for G-1-induced relaxation in the presence or absence of the adenylyl cyclase inhibitor SQ-22536. Each point represents the mean relaxation effect ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with G-1 group. *B*: concentration-response relationship for forskolin-induced porcine coronary artery relaxation. Each point represents the mean relaxation effect ± SE. Forskolin-induced relaxation began at 30 nM and reached the maximal relaxation (160%) at 3 μM.

3.3.3 PKA activity in coronary relaxation

We then tested the effect of PKA, a direct downstream target protein of cAMP, on GPER-mediated relaxation. First, we pretreated the arterial rings with Rp-8-CPT-cAMPS (5 μM), a membrane-permeant, phosphodiesterase-resistant, and cAMP-competitive PKA inhibitor. G-1-induced coronary artery relaxation was greatly inhibited by Rp-8-CPT-cAMPS (33.4% inhibition; Fig. 11Aand Table 3). To further confirm this effect of PKA, we used another PKA inhibitor, the endogenous PKA inhibitor PKI (14–22) amide (myristoylated). PKI is a small protein that contains a PKA pseudosubstrate

sequence. This pseudosubstrate binds to the substrate-binding site of PKA and thus prevents substrate binding and hence, is highly specific for PKA. The myristoylation increases its membrane permeability. Again, the relaxation effect of G-1 was significantly attenuated by inhibiting PKA activity with PKI (14–22) amide (5 μM), the G-1 concentration-response curve shifted to the left with EC50 at 278 nM (Fig. 11B and Table 3). We then used membrane-permeant PKA agonist 6-Bnz-cAMP to mimic the effect of G-1 on coronary relaxation. Like G-1, 6-Bnz-cAMP induced a concentration-dependent relaxation response. However, the relaxation occurred at a much higher concentration (EC50 = 145.3 μM) compared with the parallel G-1 group (EC50 = 27 nM) (Fig. 9A and 11C), but it caused an almost complete relaxation (96.3%) compared with a maximal G-1-induced relaxation of 44.5% (Table 3).

To confirm that G-1 indeed stimulates PKA, we measured PKA activity by applying the PepTag protein kinase assay in cultured porcine coronary artery SMCs at passage 3 (68), taking the advantage of a high concentration of protein extract from culture cells. As expected, G-1 (100 nM) raised PKA activity twofold compared with the control (Fig. 11D). To test whether the result is receptor specific, G36, an antagonist of GPER, was applied (44). G36 (1 μM) completely blocked the effect of G-1 (100 nM) and restored PKA activity to the control level (Fig. 11D), suggesting that the increased PKA activity is indeed induced by GPER activation. Taken together, with results from our tension studies, these findings demonstrate that PKA is involved in GPER-mediated coronary relaxation.

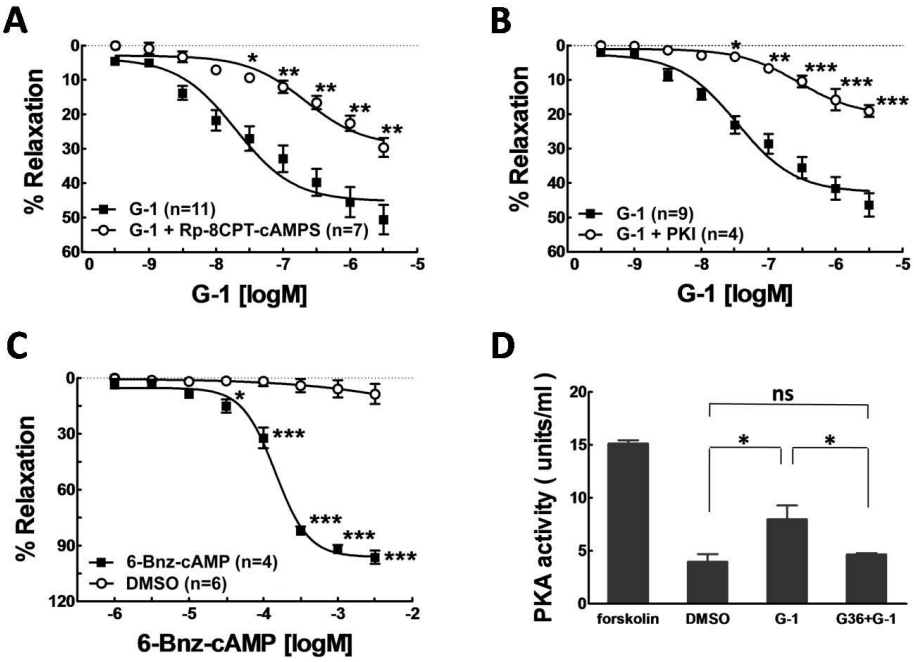

Figure 11. PKA is involved in the relaxation effect of G-1. *A*: concentration-response relationship for G-1 in the presence or absence of 8-(4-chlorophenylthio)adenosine 3′,5′-cyclicmonophosphorothioateRp-isomer (Rp-8-CPT-cAMPS; 5 μM), a cAMP analog acting as an inhibitor of PKA by competitively binding the cAMP-binding sites. Each point represents the mean relaxation effect ± SE. *B*: concentration-response relationship for G-1 in the presence or absence of myristoylated PKI (14–22) (5 μM), the membrane-permeable version of the specific PKA inhibitor PKI. Each point represents the mean relaxation effect ± SE. *C*: concentration-response relationship for N6-benzoyladenosine-3′,5′-cyclic monophosphate (6-Bnz-cAMP)-induced porcine coronary artery relaxation. Each point represents the mean relaxation effect ± SE. *D*: G-1 stimulation of PKA activities in porcine coronary artery SMCs measured by the PepTag protein kinase assay. DMSO (0.1%) acted as a solvent control, forskolin (1 μM) acted as a positive stimulator control, and cells were treated with G-1 (100 nM) in the presence or absence of G36 (1 μM), a G protein-coupled estrogen receptor 1 (GPER) antagonist, for 15 min. Results are expressed as means ± SE of 3 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the G-1 group.

3.3.4 The state of the phosphorylation of MLC/MLCP

Vascular tone depends on the phosphorylation state of the smooth muscle MLC.

When MLC is phosphorylated, smooth muscle contracts; when it is dephosphorylated, relaxation ensues. The responsible phosphatase, MLCP, can be activated by dephosphorylation via inhibition of the RhoA/Rho kinase pathway by PKA (6), thus dephosphorylating MLC and causing relaxation. To gain additional perspective on the mechanisms underlying GPER-induced vasorelaxation, we tested G-1-induced changes in the phosphorylation levels of MLC and MLCP and the contribution of PKA to these alterations. The regulatory subunit of MLCP, MYPT-1, was examined at the Thr^{853}position by Western blotting. As expected, PGF2α (1 μM) clearly induced phosphorylation of MYPT-1 at Thr^{853}, the inactive state of MLCP. The increase of the p-MYPT-1 at Thr^{853} was significantly attenuated by G-1 treatment (1 μM; Fig. 12A), indicating that GPER activation reverses the inhibitory PGF2α-induced phosphorylation of MYPT1 at Thr^{853} and thus likely enhances MLCP activity. Moreover, inhibiting PKA activity with Rp-8-CPT-cAMPS reduced the effect of G-1 and largely restored the phosphorylation of MYPT-1 at Thr^{853} induced by PGF2α, suggesting that the effects of G-1 are PKA mediated.

Because of the low sensitivity of the available MLC antibodies for porcine coronary tissue, we were not successful in detecting the MLC protein band in these same tissue samples. So we used cultured porcine coronary artery SMCs as an alternative approach to enable us to have greater protein extraction and thus better detection of MLC protein. These experiments would confirm that inhibition of PKA indeed attenuates the effect of G-1 and restores PGF2α-induced inhibition of MLCP activity. Passage 3 coronary artery SMCs were treated with PGF2α (1 μM). As expected, this

increased p-MLC. G-1 pretreatment reversed the PGF2α-induced density of p-MLC levels to control levels. The PKA inhibitor Rp-8-CPT-cAMPS attenuated G-1 effects on p-MLC density (Fig. 12B).

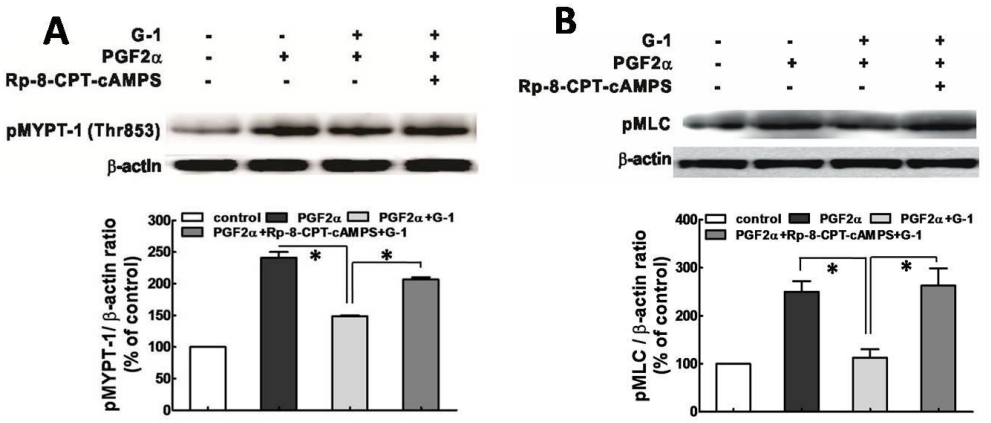

Figure 12. G-1 activates myosin light chain phosphatase (MLCP) in porcine coronary arteries. *A*: Western blot detection of myosin-targeting subunit protein-1 (MLCP regulatory subunit) phosphorylated at Thr^{853} (p-MYPT-1) in porcine coronary left anterior descending (LAD) artery tissue rings. Artery rings were collected from isometric tension studies and incubated with DMSO (solvent control), PGF2α (1 μM), PGF2α (1 μM) + G-1 (1 μM), and PGF2α (1 μM) + Rp-8-CPT-cAMPS (5 μM) + G-1 (1 μM). Top: a representative Western blot from 3 individual experiments. Bottom: bar graph of the quantitative data of the Western blot bands evaluated by densitometry. Protein amounts were normalized to β-actin, which was employed as a control for protein loading. *B*, top: representative p-MLC detection by Western blot from 3 independent experiments. Porcine coronary artery SMCs (passage 3) were serum-deprived for 18 h and then treated with drugs, as in porcine coronary tissues. B, bottom: bar graph showing the ratio of p-MLC and total β-actin, with the p-MLC and β-actin bands evaluated by densitometry. *$P < 0.05$.

The status of phosphorylation at Thr^{853} of MYPT-1 is in part a manifestation of Rho kinase activity (49). Therefore, we measured the activity of its upstream protein RhoA in human and porcine coronary artery SMCs by immunoblotting for the

Ser^{188} phosphorylation of RhoA (p-RhoA), which leads to inactivation (74), and measuring directly the activity of RhoA by G-LISA, a way to measure RhoA activation (159). As expected, the immunoblotting results showed in Fig. 13A that G-1 (1 μM) treatment of porcine coronary artery SMCs remarkably induced the phosphorylation of RhoA to an extent similar to that of forskolin (1 μM) and 6-Bnz-cAMP (10 μM) treatments, and pretreatment with PKI (5 μM) completely blocked the response of G-1 treatment, suggesting that activation of GPER inactivates RhoA activity. Consistently, G-LISA RhoA activity measurements in both human and porcine coronary artery SMCs confirmed this response of G-1. Cells were incubated with 10% fetal bovine serum to induce RhoA activity per the manufacturer's (Cytoskeleton) recommendations. Serum increased RhoA activity in both human (~2.5-fold; Fig. 13B) and porcine coronary artery SMCs (~3-fold; Fig. 13C). G-1 pretreatment (100 nM, 30 min) significantly inhibited both human and porcine coronary artery SMCs RhoA activity by ~50% in both types of coronary artery SMCs. The inhibitory effect of G-1 was restored to control (DMSO) levels by G36 (1 μM) pretreatment (Fig. 13B and C). Taken together with our vasoreactivity data and Western blot analysis, these findings strongly suggest that GPER activation mediates coronary artery relaxation through activation of MLCP via inhibition of RhoA activity by PKA.

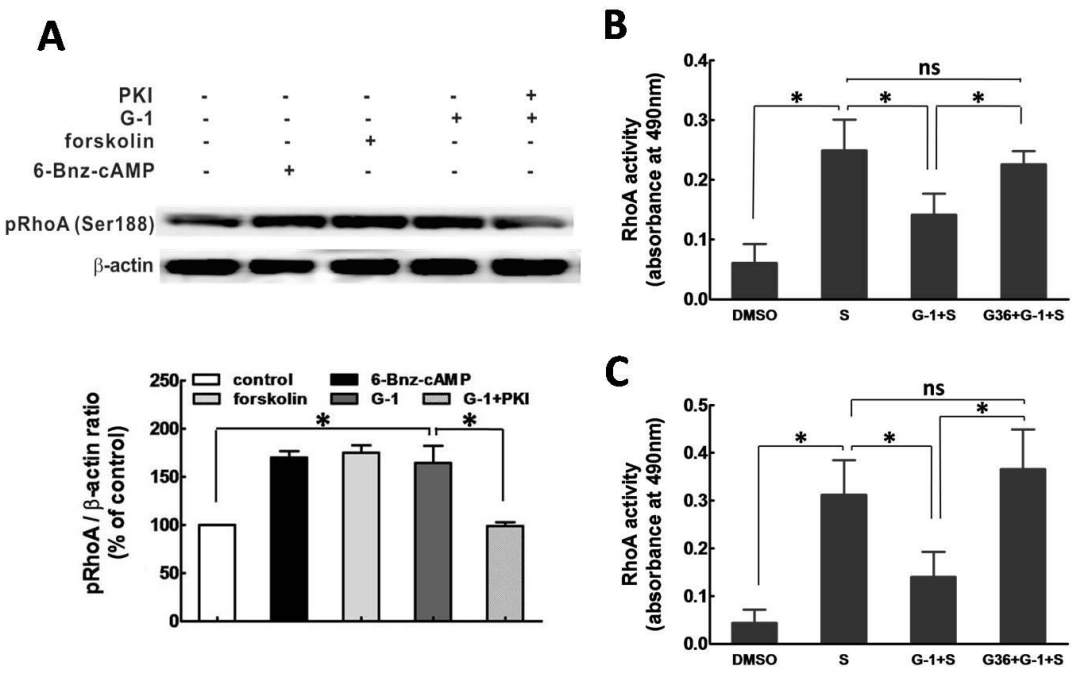

Figure 13. G-1 induces phosphorylation of RhoA and attenuates RhoA activities in coronary artery SMCs. Human and porcine coronary artery SMCs were serum-deprived for 18 h before different drug treatments. *A*: Western blot detection of phosphorylation of RhoA (p-RhoA) at Ser^{188} in porcine coronary artery SMCs (passage 3). Cells were treated for 10 min with 0.1% DMSO as solvent control, 6-Bez-cAMP (10 μM), forskolin (1 μM), G-1 (1 μM), and PKI (5 μM) + G-1 (1 μM) (n = 3). Top: a representative of p-RhoA Ser^{188}. Bottom: bar graph of the quantitative data of the Western blot bands evaluated by densitometry. Protein amounts were normalized to β-actin, which was employed as a control for protein loading. *B*: RhoA activity in human coronary artery SMCs (passage 5). Cells were treated for 2.5 min with 0.1% DMSO, 10% serum (S), 10% S + G-1 (100 nM), and 10% S + G36 (1 μM) + G-1 (100 nM) (n = 3). *C*: RhoAactivity in porcine coronary artery SMCs. Cells were received the same treatment as that of human coronary artery SMCs. *P < 0.05 (n = 4).

3.3.5 The role of A-kinase anchoring proteins

cAMP/PKA signaling is controlled temporally and spatially by A-kinase anchoring proteins (AKAPs), which confine PKA activity to relevant subsets of its substrates (13, 200). Figure 11D shows that forskolin raises PKA activity levels higher

than G-1, indicating that G-1 activates only a subpool of the total cellular PKA. Therefore, we tested whether AKAPs play a role by interacting with PKA in GPER-mediated coronary artery relaxation. In both isometric tension study and Western blotting, we applied a small molecule, 3,3′-diamino-4,4′-dihydroxydiphenylmethane (FMP-API-1), which inhibits AKAP-PKA interaction and thereby interferes with compartmentalized cAMP/PKA signaling (37). In the presence of FMP-API-1 (100 μM), G-1-induced relaxation of coronary arteries was significantly inhibited. The maximal relaxation was 22.8 ± 9.4%, reaching only about one-half of that in the presence of G-1 alone (44.51 ± 3.21%; Fig. 14A). In Western blot studies, similarly as described above, p-MYPT-1 was stimulated by PGF2α (1 μM) and attenuated by G-1 treatment (1 μM). FMP-API-1 treatment clearly reversed the effect of G-1 and restored the p-MYPT-1 level to a similar extent as treating the tissues with PGF2α alone (Fig. 14B). Together, these data suggest that interactions between AKAPs and PKA are involved in the cAMP/PKA signaling mediated by GPER in the coronary artery.

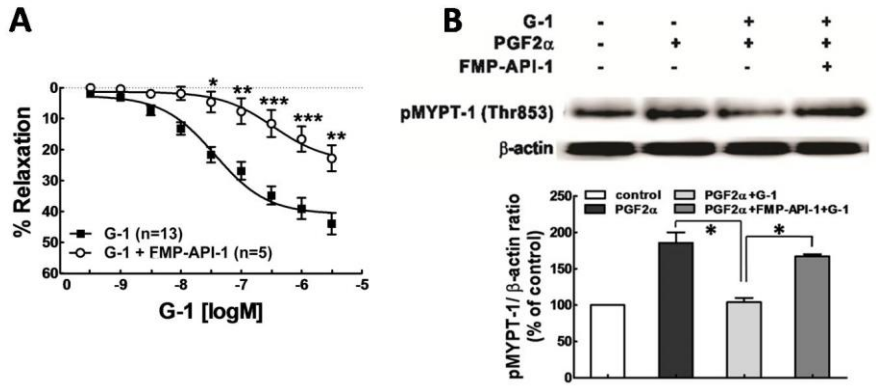

Figure 14. Inhibition of the interaction between PKA and A-kinase anchoring proteins (AKAPs) with the small molecule bis(3-amino-4-hydroxyphenyl)methane (FMP-API-1) inhibits G-1-induced relaxation of precontracted porcine coronary arteries and activation of MLCP. *A*: concentration-response relationship for G-1-induced relaxation in the absence or presence of FMP-API-1 (100 μM). FMP-API-1 attenuates the relaxation effect of G-1 compared with the G-1 group. *B*: Western blot detection of phosphorylation of MYPT-1 at Thr^{853} in LAD artery tissue rings collected from isometric tension studies. Tissue rings were incubated with DMSO (0.1%, as solvent control), PGF2α (1 μM), PGF2α (1 μM) + G-1 (1 μM), and PGF2α (1 μM) + FMP-API-1 (100 μM) + G-1 (1 μM). Representative Western blot of 3 individual experiments. Densitometric analysis of the Western blot experiments normalized to the protein loading control β-actin. *P < 0.05; **P < 0.01; ***P < 0.001.

3.4 DISCUSSION

Convincing evidence has shown that GPER exerts cardiovascular protective effects (78, 158, 179). However, knowledge of GPER downstream signaling events in the cardiovascular system is lacking. The current study demonstrates for the first time that GPER activation increases cAMP production and activates PKA. With the involvement of AKAPs, PKA inhibits RhoA activity and thereby activates the MLCP, which in turn dephosphorylates MLC and thus causes coronary artery relaxation.

Although estrogen was first found to enhance cAMP production in human coronary arteries 20 years ago (151), the mechanism whereby estrogen elevates cAMP

levels has remained undefined. Recently, studies of nonvascular cells have linked GPER to increased cAMP production. Filardo et al. (56) reported that when GPER-expressing breast cancer cells (i.e., MCF-7 cells with ERα and ERβ present and SKBR3 cells devoid of ERα or ERβ expression) were treated with estrogen, AC was stimulated and caused cAMP-dependent signaling. On the other hand, estrogen treatment of low-level GPER-expressing breast cancer cells (MDA-MB-231 cells expressing ERβ) failed to exhibit cAMP production (56). However, transfection of GPER into MDA-MB-231 cells greatly increased estrogen-stimulated cAMP production in these cells (56). The increase of cAMP attenuated ERK1/2 activity in those breast cancer cells (56). Later, Thomas et al. (216) transfected HEK-293 cells with the GPER gene and showed that estrogen treatment of the cells resulted in activation of a stimulatory G protein that was coupled directly to GPER and increased AC activity. Tamoxifen or ICI-182,780 (anti-estrogens now designated as GPER agonists) was found also to increase cAMP production in both of these studies (56, 171, 216). The effect of the GPER enhancing cAMP production was confirmed in zebrafish oocytes, which are involved in the control of fish oocyte meiotic arrest (171). In addition, both G-1 and estrogen greatly increased cAMP content along with insulin release in mouse pancreatic islets (8). While this article was in review, Lindsey et al. (128) reported that both endothelial release of nitric oxide and cAMP were involved in GPER-mediated vasorelaxation in rat mesenteric arteries. However, our previous study demonstrated that activation of GPER-induced porcine coronary artery relaxation is endothelium independent (235). Apparently, there is heterogeneity in GPER-signaling mechanisms among target vessels and cell types (e.g., nitric oxide

dependent vs. nitric oxide independent) (78). Our current studies revealed that low concentrations of G-1 (e.g., 10 nM) increased cAMP production in coronary smooth muscle cells and caused significant coronary artery relaxation. The stimulatory effect of G-1 on cAMP production paralleled its ability to relax coronary arteries in a concentration-dependent manner. Thus, these experiments are highly indicative of cAMP involvement in the vascular relaxation effect of G-1.

Use of selective cAMP analogs enabled us to test the role of cAMP and its major downstream target PKA in isometric tension studies. Our observation that the AC inhibitor SQ-22536 significantly inhibited G-1-induced coronary relaxation suggested that cAMP mediates GPER-induced relaxation. To verify a role for cAMP in mediating coronary relaxation, we applied: *1*) the AC activator forskolin to stimulate cellular cAMP production without receptor involvement and *2*) the cAMP analog 6-Bnz-cAMP to activate PKA directly. All concentrations of forskolin and higher concentrations of 6-Bnz-cAMP caused a much stronger concentration-dependent relaxation than did G-1. There are several potential explanations for this difference: *1*) forskolin is a more potent cAMP activator compared with G-1, as our results showed in Fig. 9, and *2*) forskolin has additional effects such as inhibiting the activity of ERK1/2 (202). ERK1/2 has been reported to cause dephosphorylation of caldesmon and inhibition of actin and myosin interaction (46). In addition, 6-Bnz-cAMP has an increased metabolic stability against phosphodiesterases compared with cAMP (36). Furthermore, activation of GPER might trigger multiple signaling pathways, some of which could counteract cAMP/PKA signaling. For example, Filardo and colleagues (53, 56) reported that estrogen activation

of GPER induced both cAMP production and transactivation of the epidermal growth factor (EGF) receptor through the release of pro-heparan-bound EGF from the cell surface and increased ERK1/2 activity; however, the increase of cAMP production attenuated the ERK1/2 activity a few minutes afterward.

The importance of PKA in GPER-mediated coronary relaxation was further confirmed in our study by applying two highly specific inhibitors: PKI (14-22) (myristoylated), a membrane-permeable PKA inhibitor that binds to the catalytic subunits of PKA (64, 110); and Rp-8-CPT-cAMPS, a potent competitive inhibitor of PKA that functions as a cAMP antagonist with high resistance against mammalian phosphodiesterase (36). Consistently, both of these PKA inhibitors exerted significant inhibition of G-1-induced coronary artery relaxation. These findings strongly suggest that PKA mediates GPER/cAMP downstream signaling. However, we cannot completely dismiss the possibility of the contribution from the alternative cAMP target Epac (exchange protein directly activated by cAMP), which functions as guanine nucleotide exchange factors for the small G protein Rap and plays a role in cardiac contraction and vascular permeability (65). It is possible that both PKA and Epac are involved in the signaling mediated by GPER in coronary artery relaxation. Currently, the role of the Epac/Rap signaling in GPER-mediated coronary relaxation is under investigation in our laboratory.

Some evidence suggests that ion channels are involved in PKA-mediated vascular smooth muscle relaxation through decreasing intracellular Ca^{2+} levels. Urocortin-induced, endothelium-independent rat coronary artery relaxation was shown

to be mediated primarily through PKA-dependent BK_{Ca} channels opening (93). Under normoxic conditions, forskolin or a selective PKA agonist opened BK_{Ca} channels in pulmonary artery smooth muscle cells, and these effects could be attenuated by hypoxia (9). In rat aortic smooth muscle, however, all potassium channels were reported to be responsible for about 80% relaxation induced by the ERβ activator diarylpropionitrile (ERβ selective ligand) and mediated by PKA (219). In rat mesenteric artery, vasoactive intestinal polypeptide caused relaxation through PKA-stimulated K_{ATP} channels (233). Store-operated Ca^{2+} entry was also demonstrated to be involved in coronary artery relaxation mediated by a PKA-dependent mechanism under urocortin treatment (201). Thus, it seems that ion channels, especially BK_{Ca} channels, play an important role in PKA-mediated vascular smooth muscle relaxation by lowering intracellular Ca^{2+}. Our previous work demonstrated that BK_{Ca} channels are involved in G-1-induced porcine coronary artery relaxation. Therefore, we speculate that BK_{Ca} channels might be involved in the PKA-dependent mechanism of G-1-induced coronary artery relaxation. However, future study is needed to validate this speculation.

Studies also have demonstrated that cAMP/PKA signaling may inactivate RhoA by phosphorylating RhoA at Ser^{188}, thereby releasing the inhibitory effect of RhoA and Rho kinase on MLCP, and therefore allow MLCP to dephosphorylate MLC and relax vascular smooth muscle independent of intracellular Ca^{2+} level (74, 182). MLCP activity depends largely upon the phosphorylation status of its regulatory subunit MYPT-1 at Thr^{696} and Thr^{853}. When one or two of these sites is/are dephosphorylated, MLCP will be activated to dephosphorylate MLC and induce relaxation (106). The increased Rho

kinase activity by various vasoconstrictors elevates phosphorylation of MYPT-1 at Thr^{853} but not at Thr^{696} (49, 109). Although phosphorylation of MYPT-1 at Thr^{696} is insensitive to stimuli by most agonists, it is often spontaneously phosphorylated under resting conditions (106, 109). Therefore, we only studied the phosphorylation of MYPT-1 at Thr^{853}. Our results showed that G-1 treatment induced phosphorylation of RhoA at Ser^{188} to a similar extent as that of forskolin and 6-Bnz-cAMP, attenuated serum-induced RhoA activity in human and porcine coronary smooth muscle cells, and inhibited PGF2α-induced phosphorylation of MYPT-1 at Thr^{853} in porcine coronary artery and that the PKA inhibitor Rp-8-CPT-cAMPS reversed the inhibition effect of G-1. Similarly, in porcine coronary artery SMCs, G-1 treatment decreased phosphorylation of MLC, which was reversed by Rp-8-CPT-cAMPS. These results indicate that cAMP/PKA is involved in the response to G-1, and the result that G-1 increased PKA activity in porcine coronary artery SMCs further confirmed this notion. Consistent with our results, it was reported previously that forskolin and db-cAMP, a PKA activator, inhibited a KCl-induced increase in MYPT-1 phosphorylation at Thr^{853} and the phosphorylation of MLC, thus relaxing femoral arteries from adult New Zealand white rabbits (176). Taken together, these findings suggest that through cAMP/PKA signaling, G-1 induced activation of MLCP, which resulted in dephosphorylation of MLC in coronary artery SMCs.

cAMP/PKA signaling is compartmentalized and functions locally by anchoring to the AKAPs (13, 200). Consistent with this notion, a recent study indicated that the classic estrogen receptors ERα and ERβ are colocalized with AC in the caveolae of

vascular SMCs from rat mesenteric arteries and that estrogen increases cAMP production locally, not globally, in these cells (104). AKAPs are signaling scaffolds that contribute to cAMP signaling in various aspects. They can bind to AC to regulate cAMP synthesis and sequester phosphodiesterases to break down this second messenger locally. In addition, they tether PKA to specific subcellular sites, thereby focusing the activity of PKA toward relevant substrates (13, 166). In vascular SMCs, studies have shown that the AKAP complex regulates specific vascular SMC functions, such as inhibition of cell migration, by causing localized changes in actin dynamics and reduction in cell proliferation and neointimal hyperplasia by stimulating cAMP-induced transcription and increasing the levels of the cyclin-dependent kinase-2 inhibitor p27kip1 (96, 186). In addition, the localization of PKA through binding to AKAPs facilitates stimulation of L-type Ca^{2+} channels in rabbit portal vein SMCs by endogenous PKA (239). Our novel findings from the current studies reveal that the localization of PKA by binding to AKAPs relaxed coronary smooth muscle through the inhibition of RhoA/Rho kinase (ROCK) signaling. We showed that the small molecule inhibitor of AKAP-PKA interactions, FMP-API-1 (37), significantly inhibited G-1-induced coronary relaxation. Furthermore, FMP-API-1 blocked the inhibitory effect of G-1 on the phosphorylation of MYPT-1 at Thr^{853}, which is the target site of ROCK. These results suggest that the binding of PKA to AKAPs is required for GPER-mediated coronary artery relaxation. The relevant isoforms and their localization remain unknown. Their identification is beyond the scope of this study. Candidates involved in controlling relaxation are the

three AKAPs reportedly expressed in vascular SMCs: AKAP75 (a membrane AKAP) (80), AKAP5, and gravin (AKAP12) (186).

In conclusion, we have shown for the first time that GPER-mediated porcine coronary relaxation involves localized cAMP/PKA signaling to release the RhoA/ROCK inhibitory effect on MLCP, resulting in decreased phosphorylation of MLC (Fig. 15). This has implications for GPER regulation of vascular tone in health and disease. Our findings now provide a molecular basis for developing new compounds that better target estrogen signaling for a variety of clinical applications.

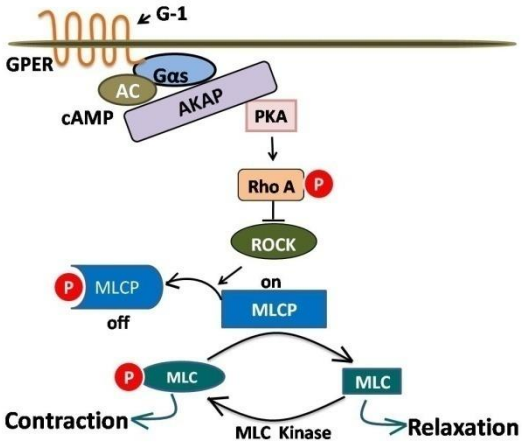

Figure 15. Proposed mechanism of GPER-mediated porcine coronary artery relaxation signaling. When GPER is activated by agonist G-1, it activates the G protein Gs, which in turn stimulates adenlylyl cyclase (AC), thus increasing cAMP production and activation of its effector PKA; PKA phosphorylates RhoA at Ser^{188}, thereby inhibiting RhoA and its effector Rho kinase (ROCK). This activates myosin light chain phosphate (MLCP) by increasing dephosphorylated MYPT-1, leading to dephosphorylation of MLC20 and relaxation.

CHAPTER IV

ACTIVATION OF G PROTEIN-COUPLED ESTROGEN RECEPTOR 1 INDUCES CORONARY ARTERY RELAXATION VIA EPAC/RAP1-MEDIATED INHIBITION OF RHOA/RHO KINASE PATHWAY IN PARALLEL WITH PKA

4.1 INTRODUCTION

Cardiovascular disease (CVD) has become the leading cause of death in the world, overtaking infectious diseases as the first cause of death in developing countries and joining industrial countries (29). Compared to men, childbearing women have lower incidence of CVD and that was credited to estrogen. Epidemiological studies have shown that the onset of CVD in pre-menopausal women occurs approximately 10 years later than that in men and myocardial infarction occurs 20 years later. However, after menopause, the risk of coronary heart disease increase was 10-fold, compared to only a 4.6 fold increase in the same age groups in men (50). Again the decrease in estrogen of menopausal women has been considered a major contribution to this increase. Evidence from earlier observational studies favored the concept that menopausal hormone treatment (MHT) protects against CVD. However, the Women's Health Initiative (WHI) MHT trials failed to demonstrate the estrogen's CVD benefit, with increased CVD incidences for menopausal hormone treatment versus placebo (92, 134). Taking the age factor into consideration, data from WHI suggests that younger/recently menopausal

women may have a better risk-benefit ratio than older/remotely menopausal women. In this regard, WHI women averaged 63 years of age, approximately12 years postmenopausal (81). Nevertheless, older menopausal women are at the highest risk of CVD and there is no "ideal" treatment available for this population yet.

A highly novel and promising therapeutic target for treating cardiovascular disease is the recently-identified membrane-associated, G-protein-coupled estrogen receptor 1 or GPER (78). GPER activation reduces blood pressure, heart and brain infarct extent (45, 238), and relaxes peripheral blood vessels (75, 127). Moreover, selective activation of GPER relaxes porcine coronary arteries (79, 144, 236). The mechanism of GPER-mediated vascular relaxation is, however, far from clear. As a typical G-protein-coupled receptor, GPER has been reported coupling to Gs, activates adenylyl cyclase, and increasec AMP production in GPER transfected HEK293 cells and human coronary artery SMCs (53, 234). Our recent work has demonstrated that cAMP/PKA signaling is involved in GPER-mediated relaxation. In human and porcine coronary artery SMCs, GPER activation increased cAMP production and activated PKA activity which in turn phosphorylated RhoA, thus, inhibited RhoA activity, resulting in activation of the myosin light chain (MLC) phosphatase (MLCP) and dephosphorylation of MLC (234).

The newly-discovered target of cAMP, Epac, exchange proteins directly activated by cAMP, is known to be a novel downstream mechanism for cAMP to govern signaling in the cardiovascular system and other tissues (141, 189), and its primary function is to act as a guanine nucleotide exchange factors (GEF) for Rap

GTPases -- which act as molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state (41). It has been reported that Epac agonist induced pulmonary and portal vein relaxation by activation of MLCP via Rap1 inhibition of Rho Kinase activities (241). In this study, we explored the role of Epac and its downstream signaling in mediating GPER-induced coronary relaxation.

4.2 MATERIAL AND METHODS

4.2.1 Tension studies

Fresh porcine hearts were obtained from local abattoir, K&C Meat Processing, immediately placed in cold Dulbecco's Phosphate Buffered Saline (Sigma) and transported back to the laboratory. Left anterior descending (LAD) coronary arteries were dissected from the fat and connective tissue, and cut into rings (axial length ~ 5 mm) to be used in isometric contractile force recordings. Arteries were endothelium-denuded to eliminate effects of endothelium-derived vasoactive factors. Only the rings with successful endothelium denudation were used, which was confirmed by the absence of relaxation to bradykinin (100 nM) exposure. Arterial rings were mounted on the two wires of isometric myographs (Danish Myograph Technology)filled with 10-ml modified Krebs-Henseleit buffer (in mM): 122 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 11.5 glucose, pH 7.2, bubbled with 95% O_2–5% CO_2 (pH 7.4) at 37 °C. One wire was connected to a force-displacement transducer and the other to a stationary micrometer. The equilibrate time for the preparations in Krebs-Henseleit buffer was 90 min. The optimal resting tension was set at 20mN in the first 30

min by gradually stretching the artery rings as in our previous work (234, 235). Isometric tension was recorded by using the LabChart data acquisition system (AD Instruments) on a PC computer. The preparations were contracted, washed and allowed to relax to basal tension for 3 times with PGF2α (1 μM). Then PGF2α (1 μM) was used to induce a stable contraction and G-1 was added in a cumulative manner by increasing the concentration in log increments. Pharmacological inhibitors were applied 30 min prior to measurement of a complete G-1or other vasodilators effects. In each set of experiments, one ring was exposed only to the constrictor agent PGF2α (1 μM) and vehicle as time control for potential fading of the contractile response. The vehicle (dimethylsulfoxide, DMSO) was added in cumulative manner by adding the identical amount of DMSO used for each concentration of G-1, 8-(4-chlorophenylthio)-2-O-methyladenosine-3,5-cyclicmonophosphate, 8-CPT-2Me-cAMPS (007). The total amount of vehicle was less than 0.1%. Relaxation responses were calculated as the % reduction in tension at each G-1 concentration from the precontracted state.

4.2.2 Vascular smooth muscle cell culture

Human coronary artery smooth muscle cells (SMCs) were purchased from Life Technologies Corp and maintained in SmGM at 37ºC in a humidified 5% CO_2 incubator. The primary porcine coronary artery SMCs culture was established as described previously (226, 227). Coronary arteries were isolated from porcine hearts. After removing the endothelial layer with a cotton-tipped swab, the media layer was dissected free from the adventitia. The medial layer was then cut into small pieces and then moved into a 25-cm^2 tissue culture flask containing 10 ml dissociation medium (in mM): 110

NaCl, 5 KCl, 2 $MgCl_2$, 0.16 $CaCl_2$, 10 HEPES, 10 $NaHCO_3$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 0.48 EDTA, 10 taurine, and 10 glucose, in addition of 24 mg elastase, 6 mg collagenase, and 15% bovine serum albumin. After gently shaking at 37^oC for 3 hours, cells were then dispersed by gentle trituration and centrifuged at 800 rpm for 5 min. The pellet was resuspended with coronary artery SMC medium, SmGM (Lonza Corp.) and cells were seeded in a 25-cm^2 tissue culture flask coated with 1% gelatin. The purity of porcine coronary artery SMCs was verified by positive staining with smooth muscle-specific α-actin (120). Passage 3 and 4 of porcine and passage 5 of human coronary artery SMCs were used in this study.

4.2.3 Western blot

Phosphorylation of MYPT-1 (pMYPT-1), the regulatory subunit of MLCP, from porcine coronary artery tissue lysates, phosphorylation of MLC (p-MLC) and phosphorylation of vasodilator-stimulated phosphoprotein (pVASP) and p-RhoA from porcine coronary artery SMC lysates were detected with Western blot analysis as previously described (234). Briefly, arterial rings were prepared as in isometric tension studies and mounted in the chamber of the myograph, equilibrated, and contracted three times with PGF2α(1 μM) as described above. Artery rings were pretreated with agonist with or without antagonists for 30 min before exposed to PGF2α (1 μM). Then the ring tissues were collected at the point of reaching contraction plateau and were snap frozen in liquid nitrogen. Tissues were pulverized (Fisher Scientific) and lysed in homogenization buffer, the composition of the buffer is (mM): 50 Tris–HCl, 0.1 EGTA and 0.1 EDTA, with 0.1% SDS, 1% NP-40, and 0.1% deoxycholic acid. For MLC20 and

VASP phosphorylation detection, porcine coronary artery SMCs were treated with agonists and antagonists as indicated in the results. Then the cells were harvested and homogenized. Protein concentrations were determined by using Detergent-compatible colorimetric assay kit (Bio-Rad). Proteins were separated by using a Mini Protean II SDS-PAGE gel kit (Bio-Rad) according to the manufacturer's instructions.

After separation, proteins were transferred to Hybond enhanced chemiluminescence (ECL) membrane (Amersham Pharmacia Biotech) with a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at constant voltage of 100 for 1 hour. Then membranes were blocked with 5% fat free milk for 1 hour at room temperature and rinsed with Tris-buffered saline (TBS)-Tween (TBST) three times, each time is 5 minutes. Membranes were then probed with specific primary antibodies in TBST containing 5% fat free milk overnight at 4°C (dilution p-MYPT-1, Thr^{853} p-MYPT-1, 1:1000, Santa Cruz Biotechnology ; p-MLC , Ser^{19},1:1000, Cell Signaling; p-VASP, Ser^{157}, 1:1000,Cell Signaling; p-RhoA, Ser^{188}, 1:200, Santa Cruz Biotechnology). The β-actin primary antibody (dilution: 1:2000, Santa Cruz Biotechnology) was used for protein loading control in the protein band densitometry analysis. Experiments were performed at least in triplicate, and mean values ± SE were calculated and made graph.

4.2.4 Rap 1 activation assay

The Rap1 activation assay is based on the differential affinity of Rap1-GTP and Rap1-GDP for the Rap binding domain of Ral GDS as described in the manufacturer's protocol. Rap1 activity was determined by using a Rap1 activation kit (Upstate Biotechnology). Briefly, porcine coronary artery SMCs were treated with G-1 (1 μM)

between 2 and 15 min with or without Farnesyl Thiosalicylic Acid (FTS, 100 μM), a potential Rap1 inhibitor (149) and 007 (50 μM) for 5 min. Cell lysates were incubated with glutathione agarose beads pre-coupled to GTPγS. Following the 45mins incubation at 4°C, beads were rinsed three times with ice-cold lysis buffer and proteins were eluted from the beads in Laemmli reducing sample buffer and were probed with anti-Rap1 polyclonal antibody by Western blot as described above.

4.2.5 RhoA activation assay

Porcine coronary artery SMCs, passage 2-3, were serum- deprived 24 hour when cells reached approximately 50% confluence. After incubated with 0.1 % DMSO (as solvent control), PGF2α (1 μM), PGF2α (1 μM) +G-1 (1 μM), and PGF2α (1 μM) +G36 (5 μM) + G-1(1 μM), cells were harvested, centrifuged at 4°C and10 kg for 2 min and the supernatant was discarded. Active Rho A- GTP activity was measured by using with RhoA G-LISA kit (Cytoskeleton), a direct measurement of RhoA activity (159), according to the manufacturer's instructions. Briefly, Cell lysates were equalized according to the total amount of the proteins and loaded into a Rho-GTP binding 96 well plate, incubated with Anti-RhoA antibody and then secondary antibody. Absorbance was read at 490 nm. Active RhoA was normalized against total RhoA in comparison of RhoA activity between groups.

4.2.6 Statistical analysis

In tension studies, comparisons were made between the presence and the absences of selective antagonist or the solvent control and vasorelaxants. Different treatments with accumulative concentrations were analyzed by using two-way analysis

of variance (ANOVA). Data was presented as mean percentage relaxations, with the standard error of the mean (SE) and the number of experiments was indicated in parentheses. Statistical differences were analyzed with Prism program (GraphPad Software Inc., San Diego, CA). In immunoblotting detection, one way ANOVA was used to detect significant differences among all treatments and student's t-testis used in paired groups. Bonferroni correction was used to correct type 1 error associated with multiple comparisons. A P value ≤ 0.05indicated a significant difference.

4.2.7 Drugs

G-1 was purchased from Calbiochem, 8-(4-chlorophenylthio)-2-O-methyladenosine-3,5-cyclicmonophosphate [8-CPT-2Me-cAMPS (007)]and 3- [5- (tert.-Butyl)isoxazol- 3- yl]- 2- [2- (3- chlorophenyl)hydrazono]- 3- oxopropanenitrile (ESI-09)arefrom Biolog Life Science Institute. PKI (14-22) amide (myristoylated) is from Enzo Life Sciences. Brefeldin A is from LC laboratories. Farnesyl Thiosalicylic Acid is from Cayman Chemical. All other chemicals were purchased from Sigma-Aldrich Corporation.

4.3 RESULTS

4.3.1 Epac and coronary artery relaxation

The pretreatment of artery rings with ARF-GEF inhibitor brefeldin A (BFA, 50 μM), a proven Epac inhibitor (241), significantly attenuated G-1-induced porcine coronary artery relaxation, the reduced relaxation was 44.90% of the total relaxation effect of G-1 (Fig. 16A, Table 4), similar to the effect of PKI (14-22) amide (5 μM), a

PKA inhibitor, in our previously study (234). When artery rings were pretreated with both BFA (50 μM) and PKI (5 μM), the inhibition of G-1-induced relaxation response reached to 76.98% (Fig. 16B, Table 4), suggesting an additive effect of Epac and PKA.As expected, the 8-CPT-2Me-cAMPS (007), an analogue highly selective for activation of Epac (190), caused a concentration-depend response of coronary artery relaxation (Fig. 16C, Table 4). However, the significant relaxation occurred at much higher concentration (EC50 = 49.4 μM), compared to G-1 (EC50 =0.027 μM), but it was similar to the effect of PKA agonist 6-Bnz-cAMP in our previous study (234).

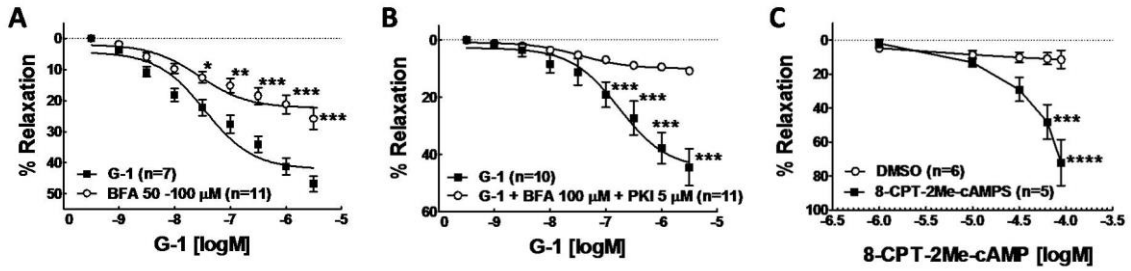

Figure 16. Epac is involved in the G-1-induced relaxation of precontracted coronary arteries. *A & B*: Concentration-response relationship for G-1-induced endothelium denuded, PGF2α (1 μM) precontracted, coronary artery relaxation, in the presence or absence of the Epac inhibitor, BFA (A); in the presence or absence of both Epac and PKA inhibitor, BFA and PKI (B). Each point represents the mean relaxation effect ± SE, *** $p<0.001$, compared to G-1 group. *C*: Concentration-response relationship for Epac agonist 8-CPT-2Me-cAMP (007)-induced porcine coronary artery relaxation. Each point represents the mean relaxation effect ± SE, *** $p<0.001$, **** $p<0.0001$, compared to the solvent control group.

Compounds	% reduction of the G-1 relaxation	% relaxation	EC_{50} (μM)
$G\text{-}1^{+}$		46.78 ± 2.52 (n=10)	0.027
BFA 50-100 μM	44.90%	25.78 ± 3.51 (n=11) ***	0.028
BFA 100 μM + PKI 5 μM	76.98%	10.77 ± 0.87 (n=11) ***	0.174
FTS 100 μM	51.95%	22.48 ± 5.09 (n=8) ***	0.23
$DMSO^{++}$		11.39 ± 5.31 (n=6)	5.56
8CPT-2Me-cAMP (007) 90 μM^{+++}		72.10 ± 13.55 (n=5) ****	49.4

Table 4. Effects of Epac signaling compounds on porcine coronary artery relaxation response to 3 μM G-1. Values are given as mean ± SE. The number of experiments is indicated in parentheses. ***P<0.001, ****P<0.0001, significant difference compared with control by using two-way ANOVA. In the upper rows (row 1 to row 4), artery rings were pretreated with each of the inhibitors and the results were compared to G-1 alone group ($^{+}$G-1). In the lower rows (row 5 to row 6), $^{++}$DMSO group was used as solvent control. $^{+++}$8CPT-2Me-cAMP was used as vasorelaxant agents and the results were compared to DMSO group.

4.3.2 Rap1 and VASP

Pre-exposure artery rings with the potential Rap1 inhibitor, Farnesyl Thiosalicylic Acid (FTS, 100 μM) (149), significantly inhibited the relaxation effect of G-1, indicating that Rap1 is functionally involved in GPER-mediated relaxation (Fig. 17A). Then we measured the Rap1 activity in the porcine coronary artery SMCs (Fig. 17B). G-1 (1 μM) treatment of the cells increased Rap1 activity at 2.5 min and 5 min, to the similar extend as the 007 (50 μM). Furthermore, the increased Rap1 activity induced by G-1 (1 μM) was completely blocked by BFA (50 μM). Together, these results suggest that as a downstream of Epac, Rap1 is involved in GPER-mediated coronary artery relaxation.

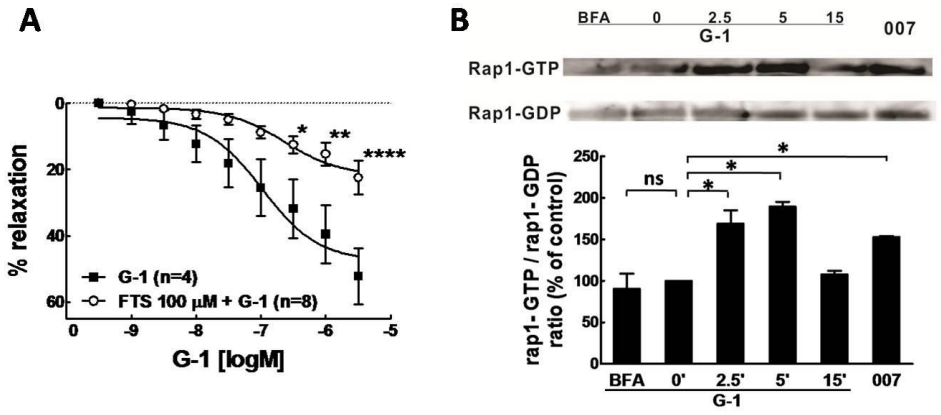

Figure 17. Rap1 is involved in G-1-induced porcine coronary arteries relaxation. *A*: Concentration-response relationship for G-1-induced relaxation in the presence or absence of the potential Rap1 inhibitor Farnesyl Thiosalicylic Acid (FTS). Each point represents the mean relaxation effect ± SE, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$, compared to G-1 group. *B*: Rap1 activity measured by using a Rap1 activation kit (Upstate Biotechnology). Porcine coronary artery SMCs (passage 4 and 5) were serum deprived for 24 hour, then treated with: BFA (50 µM) + G-1 (1 µM); G-1 (1 µM) for 0, 2.5, 5, and 15 min; 007 (100 µM). Upper panel is a representative Western blot of Rap1-GTP and Rap1-GDP from 3 individual essays. Rap1-GTP is the active form of Rap1 and Rap1-GDP is the inactive form of Rap1. The lower panel is a summary bar graph of the Rap1 Western blot bands analyzed by densitometry. Rap-GTP/Rap1-GDP ratio was normalized to control % (0 min of G-1 treatment) according to the manufacture instruction. * $p<0.05$, compared to control.

The phosphorylation of Vasodilator-stimulated phosphor protein (VASP) can be phosphorylated by PKA at Ser157, thus reflecting PKA activity (241). As expected, in porcine coronary artery SMCs, G-1 increased p-VASP, 007 did not, and the increase of p-VASP induced by G-1 was totally blocked by PKI while the antagonist of Epac, ESI-09, had no effect (Fig. 18), suggesting that Epac and PKA are two separated downstream targets of GPER and there is no cross talk between these two at the phosphorylation of VASP.

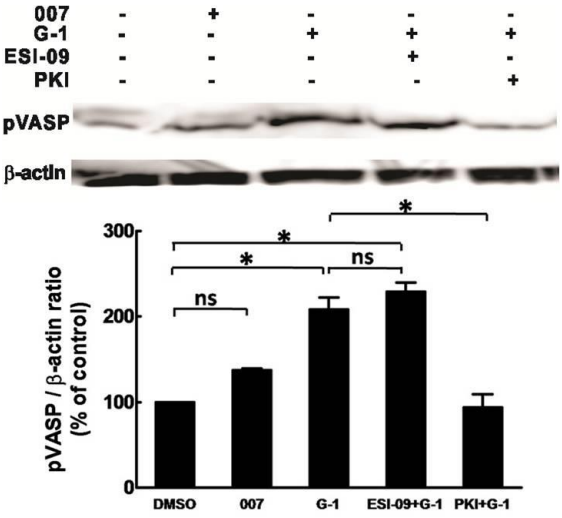

Figure 18. G-1 increases phosphorylation of VASP. Porcine coronary artery SMCs (passage 4and 5) were serum deprived for 24 hour and then treated with: 0.01% DMSO as solvent control; 007 (50 μM); G-1 (1 μM); G-1(1 μM) + ESI-09 (10 μM) and PKI (5 μM). Upper panel is a representative of Western blot for p-VASP and β–actin of 3 individual experiments. Lower panel is a summary bar graph of the Western blot band densitometry analysis. Results are expressed as mean ± SE, * $p<0.05$, compared to the solvent control.

4.3.3 RhoA activity

Previously we have shown that PKA was involved in G-1 inhibition of RhoA activity (234). Now we measured the activity of RhoA and the inactivation state of RhoA (74), the phosphorylation of RhoA at Ser^{188} (p-RhoA), by treating porcine coronary artery SMCs with 007 andESI-09 to test whether Epac plays a role in GPER-mediated inhibition of RhoA activity. The results showed that 007 (50 μM) inhibited RhoA activity to the similar extend as G-1 (1 μM) and ESI-09 (10 μM) significantly inhibited the effect of G-1 (Fig. 19A). Furthermore, the immunoblotting results showed that both 007 (50 μM) and the PKA agonist 6-Benz-cAMP (50 μM) increased p-RhoA similarly as that of G-1 (1 μM). The G-1 effect of increasing p-RhoA was significantly

inhibited by either PKI or ESI-09 (Fig. 19B). Together, these results suggest that GPER activation inhibits RhoA activity through phosphorylation of RhoA via both Epac and PKA signaling.

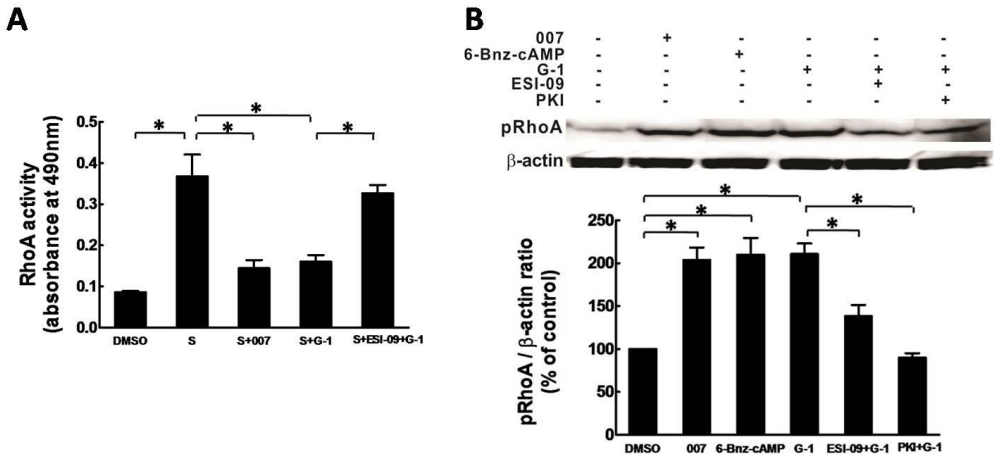

Figure 19. Epac is involved in the G-1-induced phosphorylation of RhoA and the inhibition of RhoA activities in coronary artery SMCs. Porcine coronary artery SMCs were serum deprived for 18 hour before being treated with different drugs. *A*: RhoA activity in porcine coronary artery SMCs (passage 4 and 5). Cells were treated for 2.5 min with 0.1% DMSO, 10% serum, serum + 007 (100 μM), 10% serum + G-1 (100 nM), 10% serum + ESI-09 (10 μM) + G-1 (100 nM) (n=3), * $p<0.05$, compared to groups as indicated. *B*: Western blot detection of phosphorylation of RhoA at Ser^{188} in porcine coronary artery SMCs (passage 4 and 5). Cells were treated for 10 minutes with: 0.1% DMSO as solvent control; 007 (100 μM); 6-Bnz-cAMP (10 μM); G-1 (1 μM); ESI-09 (10 μM) + G-1 (1 μM) and PKI (5 μM) + G-1 (1 μM) (n=3). Upper panel: a representative Western blot phosphor-Ser^{188} RhoA and β-actin of 3 experiments. Lower panel: Bar graph of the quantitative data of the p-RhoA bands evaluated by densitometry. Sample protein amounts were normalized to β-actin which was employed as a control for protein loading, * $p<0.05$, compared to the groups as indicated.

4.3.4 Phosphorylation of MLCP/MLC

Increased RhoA activity leads to phosphorylation of MYPT1 at Thr^{853} via Rho kinase (49, 109). In Western blotting study, G-1 (1 μM) inhibited phosphorylation of

MYPT1 at Thr^{853} induced and phosphorylation of MLC by PGF2α (1 μM) either in coronary artery rings or porcine coronary artery SMCs (Fig. 20); and the inhibition effect of G-1 was partially restored either by ESI-09 (10 μM) or by FTS (100 μM). 007 (50 μM) exerted similar inhibition effect on the phosphorylation of MYPT1 at Thr^{853} and MLC as that of G-1 (1 μM), suggesting that Epac/Rap1 signaling is involved in the GPER-mediated inhibition of p-MYPT-1 and p-MLC. In another set of experiments, both Epac and PKA antagonists were used in examining the role of Epac as well as PKA in the inhibition effect of G-1 on the phosphorylation of MYPT1 and MLC. When added separately, ESI-09 (10 μM) as well as PKI (5 μM) exerted partial restoration effect on the inhibition of phosphorylation of MYPT1 and MLC mediated by G-1; however, when both of ESI-09 (10 μM) and PKI (5 μM) were present, the G-1 induced inhibition of the phosphorylation of MYPT1 and MLC was completely restored (Fig. 21). These results suggest that Epac/Rap1 signaling along with PKA is involved in the GPER-mediated inhibition of the phosphorylation of MLCP/MLC.

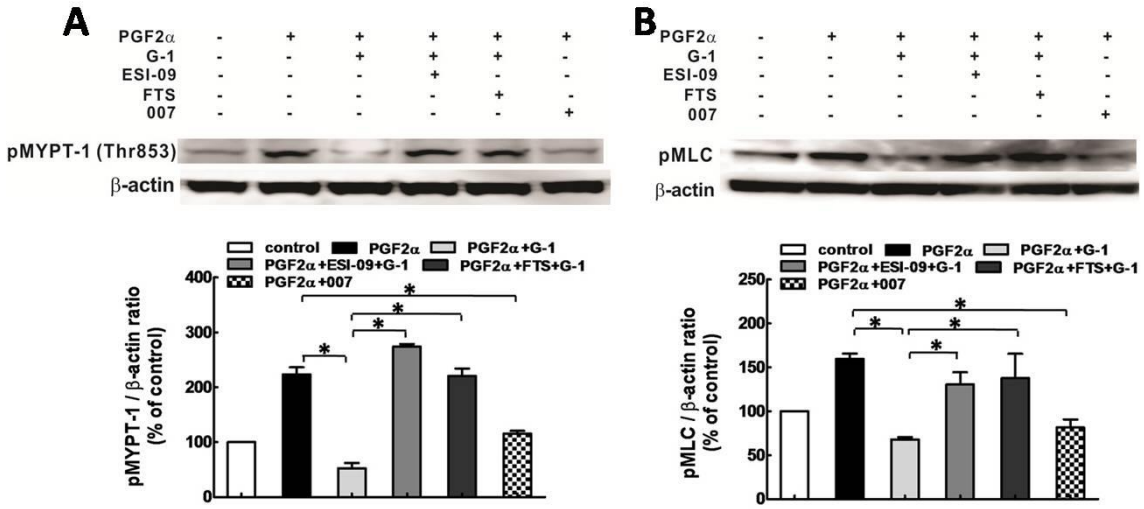

Figure 20. Epac and Rap1 are involved in the G-1 activation of MLCP in porcine coronary arteries. *A*: Western blot detection of phosphorylation of MLCP at the regulatory subunit, myosin-targeting subunit protein-1 (pMYPT-1 at Thr^{853}) in porcine coronary artery tissue rings. Artery rings in isometric tension studies were incubated with DMSO (solvent control, 0.1 %); PGF2α (1 μM); PGF2α (1 μM) + G-1 (1 μM); PGF2α (1 μM) + ESI-09 (10 μM) +G-1 (1 μM); PGF2α (1 μM) + FTS (100 μM) +G-1 (1 μM); and PGF2α (1 μM) + 007 (100 μM). Upper panel: A representative Western blot for p-MYPT1 from three individual experiments. Lower panel: Bar graph of the quantitative data of the Western blot bands evaluated by densitometry. Tissue sample protein amounts were normalized to β-actin which was employed as a control for protein loading, * $p<0.05$, compared between groups as indicated. *B*: Upper panel: the representative p-MLC detection by Western blot from three independent experiments. Porcine coronary artery SMCs (passage 4) were serum deprived for 24 hours, and then treated with drugs as in porcine coronary tissues, except that the concentration of ESI-09 was 10 μM. Lower panel: Bar graph showing the summary data of p-MLC which was normalized to total β-actin, the bands were evaluated by densitometry, * $p<0.05$, compared to the group as indicated.

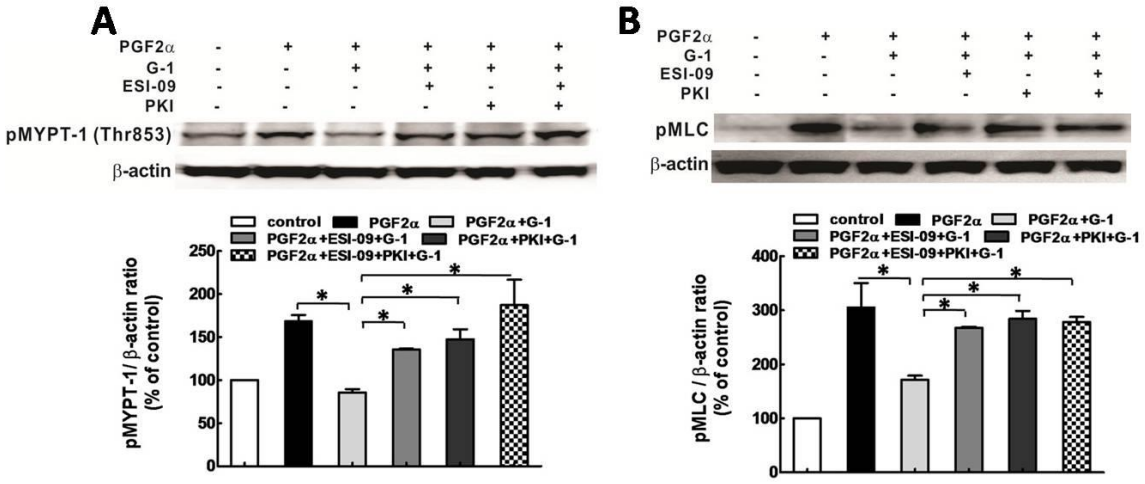

Figure 21. Epac and PKA exert additive effect on G-1-induced activation of MLCP in porcine coronary arteries. *A*: Western blot detection of pMYPT-1 at Thr^{853} in porcine coronary artery tissue rings. Artery rings were treated with: DMSO (solvent control, 0.1 %); PGF2α (1 μM); PGF2α (1 μM) + G-1 (1 μM); PGF2α (1 μM) + ESI-09 (10 μM) +G-1 (1 μM); PGF2α (1 μM) + PKI (5 μM) + G-1 (1 μM); and PGF2α (1 μM) + ESI-09 (10 μM) + PKI (5 μM) + G-1 (1 μM). Upper panel: A representative Western blot for p-MYPT1 from three individual experiments. Lower panel: Bar graph summary of the quantitative data of the Western blot p-MYPT-1 bands evaluated by densitometry. Tissue sample protein amounts were normalized to β-actin. * $p<0.05$, compared between groups as indicated in the graph. *B*: Upper panel: A representative p-MLC detection by Western blots of three independent experiments. Porcine coronary artery SMCs (passage 4) were serum deprived for 24 hours, and then incubated with the drugs as used in porcine coronary tissues, except that 10 μM of ESI-09 was used rather than 25 μM. Lower panel: Bar graph showing the summary data of p-MLC normalized to total β-actin, the bands were evaluated by densitometry, * $p<0.05$, compared to the group as indicated in the graph.

4.4 DISCUSSION

There is a growing interest in the role of GPER in cardiovascular regulation. This work is the first to define the cAMP/ Epac signaling pathway in GPER-mediated coronary artery relaxation, which is through attenuation of phosphorylation of MLCP and MLC via Rap1 inhibition of RhoA activity in parallel with PKA.

GPER activation induces vascular dilation in a variety of vascular beds (75, 127, 145, 235) and reduces tissue damages after heart ischemia-reperfusion or stroke (45, 238). However, our understanding of the mechanism of GPER-mediated vasorelaxation has just begun. Our recent work has shown that: G-1 treatment of porcine coronary artery SMCs activated adenylyl cyclase (AC) and increased cAMP production in a concentration-dependent manner; isometric tension study showed that blocking of AC inhibited G-1 induced porcine coronary artery relaxation, suggesting cAMP was involved in the GPER-mediated coronary artery relaxation. The downstream PKA signaling was then proven playing a role in GPER-mediated coronary vasorelaxation by activating MLCP via inhibition of RhoA pathway (234). Now we have found that, along with PKA, Epac/Rap1 signaling is also involved in the GPER-mediated vasorelaxation via inhibition of RhoA pathway.

The elevation in intracellular concentration of cAMP after Gs-protein-coupled receptors activation is sufficient to activate EPAC (189). Now Epac is recognized as a novel mechanism in the regulation of cardiovascular functions (141). Two isoforms of Epac, Epac1 and Epac2, have been identified (141). Epac1 is the predominant isoform found in VSM of rat aorta and mesenteric artery (183). Activation of Epac1could induce relaxation of adrenaline-contracted rat aortae (210) and phenylephrine-contracted rat mesenteric arteries (241). In the present study, G-1 induced porcine coronary artery relaxation can be significantly attenuated with ARF-GEF inhibitor brefeldin A (50 μM), which was shown to be an Epac inhibitor (241); furthermore, the Epac specific agonist 8CPT-2Me-cAMP (007) mimicked the relaxation effect of G-1. Together these findings

suggest that Epac, as a cAMP downstream, is also involved in the GPER-mediated relaxation effect; and functioned similarly as PKA mediating GPER vasorelaxation effect as reported in our previous study (234).

Epac is the best described guanine nucleotide exchange factor (GEF) in cardiovascular system, and facilitates Rap1 cycling from the (GDP-bound) inactive state to the (GTP- bound) active state (99).Rap1 as members of the Ras family are involved in the network of multiple proteins to regulate vascular endothelial cell proliferation, migration and endothelial permeability as well as vascular tone (38). Rap1b in both smooth muscle and endothelium plays a key role in maintaining blood pressure. Rap1b−/− mice developed basal tone elevation and hypertension that was probably caused by: *1)* increased contractility of smooth muscle to contractile agents, such as, thromboxane, angiotensin II or phenylephrine; *2)* defective endothelial release of dilatory nitric oxide in response to elevated blood flow (116). We tested the effect of GPER activation on Rap1 in porcine coronary artery SMCs and observed that G-1 (1 μM) markedly increased Rap1 activity between 2 and 5 minutes, to the similar extend as 007, and the increase was blocked by BFA (50 μM), suggesting that GPER activation stimulates Rap1 activity via Epac in coronary artery SMCs. The isometric tension study results showed that the inhibitor of Rap1, FTS (100 μM), significantly reduced the relaxation effect of G-1, further conformed the involvement of Epac/Rap1 in the GPER-mediated coronary artery relaxation. In vitro studies have evidenced several underlining mechanisms for Epac/Rap1induced vascular relaxation, including attenuation of RhoA activity (241) and indirect modulation of K^+ channel activity (183, 190).

Increase in RhoA activity stimulated by various vasoconstrictors leads to phosphorylation of MYPT1, at Thr^{853}, not at Thr^{696}, via Rho kinase (49, 109). The elevated phosphorylation of MYPT1 largely inhibits MLCP activity, therefore, sustaining the vascular smooth muscle contraction by keeping MLC phosphorylation from falling. On the other hand, when MYPT1 is dephosphorylated, MLCP will be activated to dephosphorylate MLC and induce relaxation (106). Evidences from other people's work and ours have shown that cAMP/PKA signaling may inhibit RhoA by phosphorylating RhoA at Ser^{188}, thereby releasing the inhibition of RhoA/Rho kinase on MLCP, thus allowing MLCP dephosphorylate MLC and cause relaxation of vascular smooth muscle independent of intracellular Ca^{2+} level (74, 182, 234). Since the phosphorylation of MYPT-1 at Thr-^{696} site is often spontaneously phosphorylated under resting conditions and is insensitive to stimuli by most agonists (106, 109), we only studied the phosphorylation of Thr^{853} of MYPT-1. Our results showed that: the antagonist of Epac, ESI-09, significantly inhibited G-1-induced phosphorylation of RhoA at Ser^{188} and restored G-1-reduced RhoA activity; Epac agonist, 007, on the other hand, increased phosphorylation of RhoA and decreased RhoA, similar to that of G-1 and PKA agonist, 6-Benz-cAMP; At the phosphorylation of Thr^{853} of MYPT1, when either PKA and Epac antagonist present, the decrease of the p-MYPT-1 mediated by G-1 was partially restored, when both present, they exerted adding effect and completely restored the p-MYPT-1. Together these findings demonstrate that Epac plays a similar role as PKA in GPER-mediated coronary relaxation via inhibition of RhoA and p-MYPT-1. Consisting with our findings are findings from rat aortic smooth muscle, gut

and airway smooth muscles, in which, Epac agonist 007 as well as the vasodilator PGI_2 analog, cicaprost, increased Rap1 activity and decreased RhoA activity (241). Activation of a plasma membrane-bound G protein-coupled receptor TGR5, known as GPBAR1 (G protein-coupled bile acid receptor1), has also been reported causing relaxation of gastric smooth muscle, which is mediated through inhibition of RhoA/Rho kinase pathway via cAMP/Epac-dependent stimulation of Rap1 (185).

The roles that Epac plays in respect to PKA in mediating cAMP signaling can be separate, opposing, or additive and synergetic, depending on the tissue or cell types and their various conditions. In renal, PKA and EPAC play separate roles and are engaged in very different downstream physiological functions in principal cells and stellate cells (51). In large number of cell types, PKA mediates pro-apoptotic response to cAMP; while Epac mediates the anti-apoptotic response (97). Similarly, in neurons, Epac and PKA play opposing roles in regulating axon guidance (152). In heart, PKA and Epac function cooperatively in neo-formation of gap-junction of rat cardiac myocytes (203); additionally, in an additive and independent manner, Epac and PKA phosphorylates phospholamban and ryanodine receptor in the regulation of cardiac function (164). However, Epac and PKA exert opposing effects on Ca^{2+}-activated force and myofilament Ca^{2+} sensitivity in those rat cardiac myocytes (30). In vascular SMCs, both PKA and Epac pathways are necessary for cell growth inhibition. Epac synergises with PKA to mediate cAMP-induced cell growth arrest (87). In cAMP-mediated vascular, gut and airway smooth muscle relaxation, Epac and PKA are involved independently in the downstream signaling (185, 241). In this study we showed that Epac and PKA exert

additive effect in mediating GPER downstream signaling at the phosphorylation of MYPT-1, although they signal in separate pathways as confirmed by the result that neither Epac agonist nor antagonist had any effect on the phosphorylation of VASP at Ser^{157}, a marker for monitoring PKA activity (241).

Ion channel recording study in rat aortic SMCs has shown that cAMP-mediated activation of Epac inhibited K_{ATP} channel activity via activation of Ca^{2+}-sensitive protein phosphatase 2B (183). A more recent study suggests that Epac mediated rat mesenteric artery relaxation by limiting Ca^{2+} entry via voltage-sensitive Ca^{2+} channels via SMCs hyperpolarization caused by: *1)* activation of BK_{Ca} channels by Ca^{2+} sparks released from ryanodine receptors, and *2)* the activation of SK_{Ca}/IK_{Ca} channels of endothelial cells (190). Our previous work has shown that G-1 treatment of porcine coronary artery SMCs opens BK_{Ca} channels. The activation of these channels requires the integrity of cytosol signaling (236). We may speculate that either PKA or Epac or both may be involved in the BK_{Ca} channels opening mechanism of GPER.

4.5 CONCLUSION

In conclusion, the results of this study confirm that GPER-mediated porcine coronary relaxation involves both Epac and PKA signaling to inhibit the RhoA/ROCK effect on MLCP, therefore, causing decreased phosphorylation of MLC and relaxation of coronary artery (Fig. 22). These findings offer clearer understanding on the role of GPER in vascular tone regulation and provide a molecular basis for GPER as a potential drug target in preventing and treating cardiovascular disease in women.

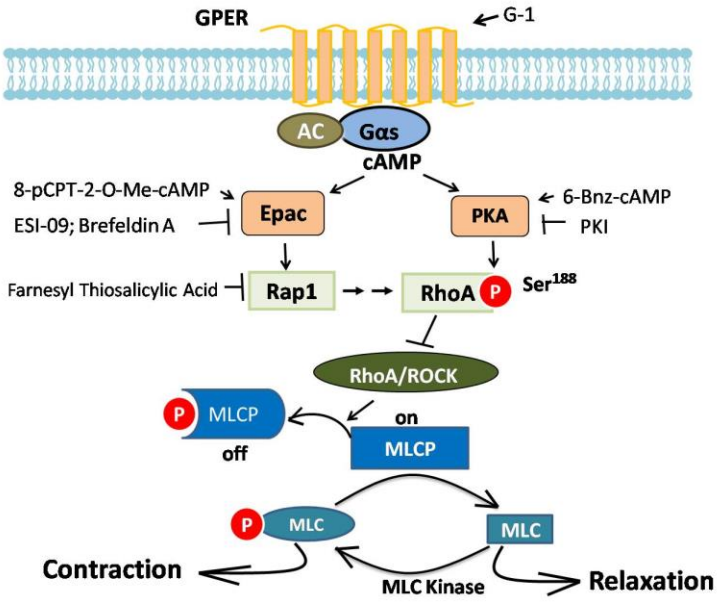

Figure 22. Proposed mechanism of Epac and PKA pathways in GPER-mediated porcine coronary artery relaxation signaling. When GPER is activated by G-1, it activates Gs protein. Gs stimulates adenlylyl cyclase (AC) and increases cAMP generation. Cyclic AMP activates both downstream targets, Epac and PKA. Epac activates Rap1, then RhoA is phosphorylated at serine 188 by both Rap1 signaling and PKA, thereby the activity RhoA and its effector Rho Kinase (ROCK) is inhibited, thus releasing the inhibition on MLCP by decreasing the phosphorylation of MYPT1, the myosin phosphatase target subunit 1 of MLCP, leading to decreased phosphorylation of MLC20 and coronary artery relaxation.

CHAPTER V

ACTIVATION OF G PROTEIN-COUPLED ESTROGEN RECEPTOR 1 ENHANCES ENDOTHELIN-1-INDUCED CORONARY ARTERIES CONSTRICTION VIA TRANSACTIVATION OF EGFR

5.1 INTRODUCTION

Cardiovascular disease continues to claim the lives of more women in the U.S. than any other health problem, with coronary heart disease (CHD) as the single leading cause of mortality (66). Although lipid-lowering statins and antiplatelet aspirin reduce the risk of CHD in men, these therapies are ineffective for women (15, 175). There is an urgent need for developing a means of preventing CHD in women. In early menopause estrogen therapy helps protect against CHD (88, 161); yet paradoxically, estrogen is associated with an increased risk of both CHD and stroke in postmenopausal women> 65 years of age (130). There is no means of effectively preventing or treating CHD in this population who are at highest risk for cardiovascular mortality and morbidity.

A highly novel and promising therapeutic target for treating cardiovascular disease is the recently-identified membrane-associated, G-protein-coupled estrogen receptor 1 or GPER (78). GPER activation reduces blood pressure, heart and brain infarct extent after ischemia-reperfusion damage or stroke (45, 238), and relaxes peripheral blood vessels (75, 127). Moreover, we have recently demonstrated that

selective activation of GPER relaxes porcine coronary arteries and targets smooth muscle cells from human coronary arteries (236). On the other hand, GPER induces vasoconstriction in the kidney (114). Thus, GPER can mediate opposite effects on blood vessels, and we propose that it is very likely that these dual actions of GPER can contribute to the seemingly paradoxical effects of estrogen in regulating coronary artery function. However, the mechanism underlining the GPER-mediated vasoconstriction is far from completed.

In human coronary microarteries, estrogen potentiated angiotensin II-induced vasoconstriction via GPER and EGFR (epidermal growth factor receptor) activation (12). Direct constriction effect of GPER activation was shown in an isolated rat kidney perfusion study, when the artery was under a pre-constriction stage, addition of GPER agonist G-1induced relaxation, however, under conditions without any vasoconstriction agent, G-1 alone caused artery contraction and the vasoconstriction may be blocked by inhibitors of EGFR and ERK1/2 (17, 114). Filardo et al. (56) also provided evidences on GPER transactivation of EGFR. They reported that in MCF-7, SKBR3 and MDA-MB-231 cells, estrogen caused attenuation of ERK1/2 activity via Gβγ-dependent transaction of EGFR by activation of GPER. But, how GPER is mediating these opposing effects of estrogen and whether GPER contributes to the paradoxical effects of estrogen in postmenopausal women remain unknown. In this study, we provide answers to these important questions by testing the hypothesis that activation of GPER enhances coronary artery contraction by the EGFR/ERK1/2 signaling pathway.

5.2 MATERIALS AND METHODS

5.2.1 Supply of coronary arteries

Porcine hearts were obtained from local abattoirs, K&C meat processing and placed into ice-cold Krebs buffer of the following composition: 131.5 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 2.5 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2 g glucose and 2 g $NaHCO_3$ in 1 L with pH 7.4 (The solution was previously oxygenated with 95% O_2-5% CO_2 for 30 minutes). Hearts were kept on ice during transport to the laboratory. Coronary arteries were dissected from porcine hearts and the protocol was in compliance with the Texas A&M University Institutional Animal Use Protocol.

5.2.2 Tension studies

The left anterior descending coronary artery (LAD) were dissected and cleaned of excess collective tissue and fat tissue. To eliminate indirect effects of endothelium-derived vasoactive factors, the endothelium were removed physically by rubbing the intimal surface and tested by observing the absence of bradykinin-induced relaxation of PGF2α induced contraction. LADs were cut into ~3 mm rings and mounted on two parallel tissue supports, with one support fixed to a stationary chamber and the other attached to a force transducer (AD instruments). Isometric contractile force was recorded on a computer using LabChart software. The tissue bathing solution was Krebs buffer, oxygenated continuously with 95% O_2-5% CO_2 and maintained at 37°C. Coronary artery rings were equilibrated for 90 min under an optimal resting tension of 20mN, and bath solution was changed every 30 min to prevent accumulation of metabolic products. After equilibration, vasoconstrictor prostaglandin (PG)F2α (1 μM) was administrated to check

rings contractility. Inhibitors were added 30 min prior to the measurement of a complete G-1 concentration-response relationship (1 to 3000 nM). Vasodilatory responses were calculated as the percent reduction in tension from the pre-contracted state.

5.2.3 Cell cultures

The method to be used to disperse cells has been described by Chamley-Campbell et al.(32). Briefly, coronary arteries were dissected, cut into pieces and placed into 3mg/ml collagenase in modified dissociation medium (110 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.16 mM $CaCl_2$, 10 mM HEPES, 10 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 0.5 mM NaH_2PO_4, 0.49 mM EDTA, 10 mM Taurine 10 mM glucose and 15% fetal bovine serum) at 37°C for 1 h, followed by 0.5mg/ml elastase for 1h in the same medium. Then the tissue pieces were dispersed into single cell with complete SmBM basal medium (Lonza) and seeded into 60-mm gelatin-coated plastic dishes with density of $2x10^5$cells/ml. Primary cultured porcine coronary artery smooth muscle cells (SMCs) and human coronary artery SMCs (Cascade Biologics) were maintained in complete SmBM basal medium. Cell cultures were kept at 37°C and under 5% CO_2 in a humidified incubator. The purity of porcine coronary artery SMCs was verified by positive staining with smooth muscle-specific α-actin (120). Coronary artery SMCs were cultured to 80% -90% confluence, and then we employed passage 3-4 for porcine coronary artery SMCs or passage 5-6 for human coronary artery SMCs.

5.2.4 Western blots

After pretreated with inhibitors and followed by different treatments in isometric tension studies, LAD coronary artery rings were snap-frozen in liquid nitrogen,

pulverized and then lysed in RIPA lysis buffer (Sigma) with protease and phosphatase inhibitors. Porcine and human coronary artery SMCs were harvested and lysed in the same solution. Protein concentrations were determined by Pierce BCA protein assay and samples were separated on precast 4%-12% Bis-Tris gels (Invitrogen) according to the manufacturer's instructions. Then proteins were transferred to PVDF membrane (EMD Millipore) at 100 V for 2 h. Membranes were blocked with 5% nonfat milk for 1 h at room temperature and then incubated with first primary antibody in TBST containing 5%nonfat milk protein for overnight at 4°C. After washing and incubating with second antibody, the membranes were ready for chemifluorescent detection. Membranes were stripped and immunoblotted with β-actin antibody for protein loading control.

5.2.5 Statistics

Vascular function studies were analyzed by different treatments (the presence or the absence of inhibitors in G-1 accumulation concentration responses, or G-1 pretreatment with the presence or absence of inhibitor in ET-1 accumulation concentration responses) using two-way analysis of variance (ANOVA) for multiply groups and Student's t-tests between paired groups. One way ANOVA was used to detect significant differences among all groups of immunoblotting detection data and student's t-testis used in paired groups. Data was expressed as means ± SE and analyzed with Prism program (GraphPad Software Inc., San Diego, CA). Bonferroni correction was used to correct type 1 error associated with multiple comparisons. A P value ≤ 0.05 indicated a significant difference.

5.2.6 Materials

Antibodies were purchased from Santa Cruz Biotechnology. Both G-1 and G36 were purchased from Azano Pharmaceuticals Incorporation. The inhibitors tested in tension studies were purchased from Tocris Bioscience.

5.3 RESULTS

5.3.1 Coronary contraction and the functional role of Gβγ

Although the activation of GPER causes stimulation of Gβγ signaling has been implicated in MCF-7, SKBR3 and MDA-MB-231 cells (56), there is no direct evidence that has shown Gβγ activation following stimulation of GPER in coronary arteries. We tested whether GPER enhances coronary contraction by applying cumulative ET-1 concentration-response relation in the presence or absence of G-1 or G-1 + G36in porcine left anterior descending coronary arteries. As shown in Fig.23A, arteries pre-incubated with G-1 (1 μM) showed an enhancement of ET-1 concentration response, a significant higher contraction force was induced compared after ET-1 concentration reached to 10 nM, compared to the control group, that is the identical concentration ofET-1 alone group, and GPER antagonist,G36 (10 μM), blocked this constriction enhance effect of G-1. The maximal contraction of G-1 pretreated artery rings were 65.59 ± 5.41 mN/mg without changing EC50 (Table 5). Next, we tested the functional role of Gβγ in GPER-mediated coronary artery contraction. The specific Gβγ inhibitor Gallein was used to block Gβγ cell signaling in these artery rings. Gallein (10 μM) markedly increased G-1-induced relaxation with maximal relaxation of 65.39 ± 2.25 %

and no change on EC_{50} (Fig.23B, Table 6). These findings from tension studies suggest that Gβγ is involved in GPER-mediated coronary contraction.

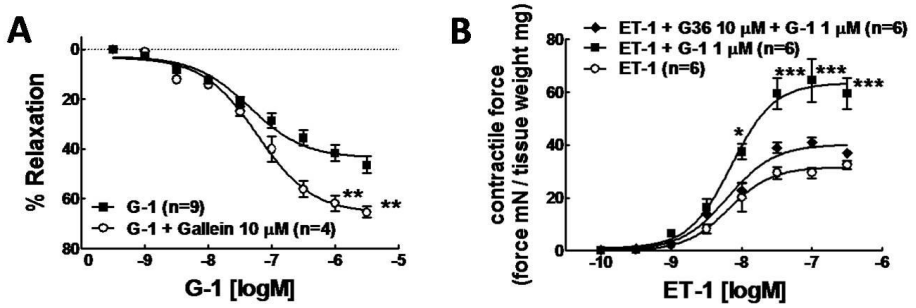

Figure 23. G-1 promotes ET-1-induced coronary arteries contraction and Gβγ is involved in G-1-enhancedvasoconstriction. *A*: concentration-response relationship for ET-1-induced contraction in the presence or absence of G-1 or G-1 + G36. Each point represents the mean contractile force± SE. Contractile force was the force generated by artery rings calibrated with artery weight with unit mN/mg. **P < 0.01 compared with ET-1 group using two-way ANOVA. *B*: concentration-response relationship for G-1-induced porcine coronary artery relaxation in the presence or absence of selective Gβγ inhibitor Gallein. Each point represents the mean relaxation effect ± SE.*P < 0.05, compared with G-1 group.

Compounds	Contractile force (mN/mg)	EC_{50} (nM)
ET-1^{+}	32.49 ± 1.62	8.59
G-1 1 μM	65.59 ± 5.41 (n=6) ***	no change
G36 10 μM + G-1 1 μM	36.99 ± 0.75 (n=6)	no change
PP2 10 μM + G-1 1 μM	28.30 ± 4.21 (n=5)	no change

Table 5. Effects of compounds on porcine coronary artery contraction response to 0.3 μM ET-1. Values are given as mean contractile force ± SE. The number of experiments is indicated in parentheses. Artery rings were pretreated with each of the inhibitors and the results were compared to ET-1 alone group ($^{+}$ET-1). ***P<0.001, significant difference compared with ET-1 alone group ($^{+}$ET-1) by using two-way ANOVA.

Compounds	% increase of the G-1 relaxation	% relaxation	EC_{50} (μM)
$G\text{-}1^{+}$		46.39 ± 3.37 (n=10)	0.034
Gallein 10 μM	40.96%	65.39 ± 2.25 (n=4) **	0.107
AG1478 5 μM	50.21%	69.68 ± 3.52 (n=8) ***	no change
PD 98059 1 μM	39.82%	64.86 ± 3.12 (n=6) **	no change

Table 6. Effects of Gβγ/EGFR/ERK1/2 signaling compounds on porcine coronary artery relaxation response to 3 μM G-1. Values are given as mean relaxation effect ± SE. The number of experiments is indicated in parentheses. Artery rings were pretreated with each of the inhibitors and the results were compared to G-1 alone group ($^{+}$G-1).**P<0.01, ***P<0.001, significant difference compared with G-1 alone group ($^{+}$G-1)by using two-way ANOVA.

5.3.2 EGFR transactivation in coronary contraction

Activation of EGFR has been implicated in many cardiovascular diseases (133), and may provide new targets for control of vascular smooth muscle cell proliferation in response to arteries injury (100). Although evidence has shown that GPER induces EGFR transactivation in breast cancer cells (55), there is no information available in vascular smooth muscle. We first tested the functional role of EGFR in tension study by employing AG1478, a selective EGFR tyrosine kinase inhibitor (98). AG1478 (5 μM) enhanced G-1-induced relaxation by more than 50% (Fig.24A, Table 6), reaching statistical significance at 30 nM through 3 μM, but there was no change in EC50 value, suggesting that EGFR is involved in GPER-mediated vascular tone regulation. Further, we tested G-1 effect on EGFR transactivation in porcine coronary artery SMCs by

detecting tyrosine phosphorylation of EGFR with Western blot. G-1 (1 μM) clearly increased EGFR phosphorylation at both 5 and 10 min., similar to EGF (10 ng/ml), while AG1478 attenuated the effect of G-1 on phosphorylation of EGFR (Fig. 24B).

Src is the upstream of EGFR transactivation mediated by GPER in breast cancer cells (55). Evidence has shown that Src is involved in modulation of coronary artery contractility (154). Thus, we determined whether Src is involved in GPER mediated potentiation of coronary artery contraction by pretreating coronary artery rings with inhibitor of Src, phosphoprotein phosphatase 2 (PP2), and measuring the cumulative ET-1 concentration response. As expect, G-1 pretreatment enhanced ET-1 induced coronary artery comparing to ET-1 group, while Src inhibitor PP2 (10 μM) blocked the enhanced contraction effect of G-1. The maximal contraction in the presence of both PP2 and G-1 was 28.30 ± 4.21 mN/mg without changing EC_{50} (Fig.24C and table 5). Taken together with vascular functional studies and Western blot analysis, these findings suggest that GPER activation potentiates coronary artery contraction through transactivation of EGFR via Src.

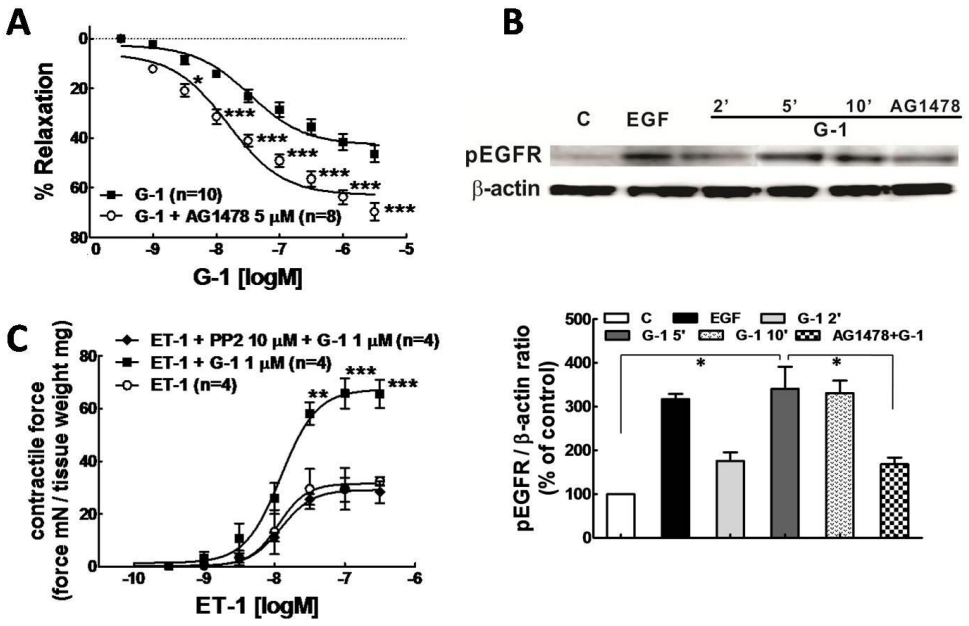

Figure 24. EGFR transactivation is involved in the coronary tone regulation of G-1. *A*: Concentration-response relationship for G-1-induced porcine coronary artery relaxation in the presence or absence of selective GPER inhibitor AG1478. Each point represents the mean relaxation effect ± SE.*P < 0.05, ***P<0.001, compared with G-1 group using two-way ANOVA.*B*: Western blot detection of phosphorylation of EGFR in porcine coronary artery SMCs. Cells were incubated with DMSO (solvent control), EGF (100ng/ml) 10min, G-1 (1μM) 2min, G-1 (1 μM) 5min, G-1 (1 μM)10min and AG1478 (5 μM) +G-1(1 μM). Top: a representative Western blot from 3 individual experiments. Bottom: bar graph of the quantitative data of the Western blot bands evaluated by densitometry. Protein amounts were normalized to β-actin, which was employed as a control for protein loading.*P < 0.05, compared to the group as indicated in the graph. *C*: concentration-response relationship for ET-1-induced contraction in the presence or absence of G-1 or G-1 + PP2. Each point represents the mean contractile force ± SE. Contractile force was the force generated by artery rings calibrated with artery weight with unit mN/mg. **P < 0.01, ***P < 0.001,compared with ET-1 group using two-way ANOVA.

5.3.3 ERK1/2 activation in coronary contraction

GPER activation can stimulate Src-related tyrosine kinase activity-dependent EGFR transactivation and then cause ERK1/2 phosphorylation in breast cancer cells

(55). Inhibition of ERK1/2 with PD98059 significantly attenuated G-1-induced vasoconstriction of rat renal arteries (114). We tested the role of ERK1/2 by applying ERK1/2 upstream MEK inhibitor PD98059 in tension study and detecting phospho-ERK1/2 in immunoblotting. In the presence of both PD98059 (1μM), G-1-induced relaxation of coronary arteries was significantly increased with maximal relaxation of 66.86 ± 3.12% (Fig. 25A, Table 6). In western blots, we tested G-1 effect on ERK1/2 activity in porcine coronary artery SMCs by detecting phosphorylation of ERK1/2 in the presence or absence of adenlyly cyclase inhibitor SQ22536. In the presence of SQ 22536 (100 μM), G-1 (1 μM) significantly increased ERK1/2 phosphorylation at all time points with the highest at 30 minutes similar to EGF (10 ng/ml). On the other side, in the absence of SQ22536, G-1 (1 μM) decreased about one and half of phospho-ERK1/2at all-time points. As expect, G36blocked G-1 stimulation effect in the presence of SQ22536 as well as the inhibition effect of G-1 in absence of SQ22536 (Fig. 25B and C). Together, these findings suggest that GPER may promote coronary artery contraction via ERK1/2 activation.

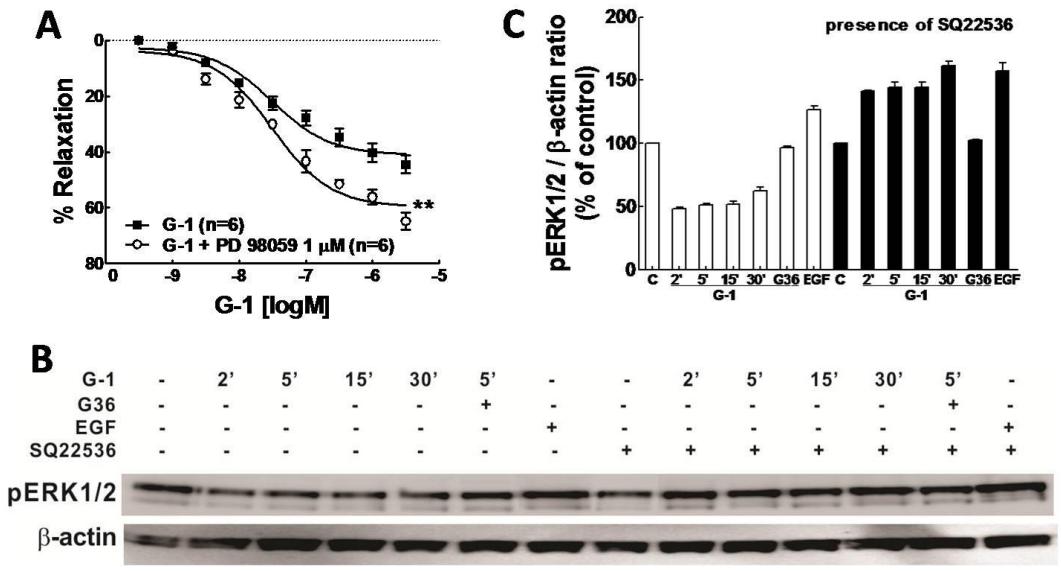

Figure 25. G-1 activates ERK1/2 activity in porcine coronary arteries and porcine coronary artery SMCs in the presence of SQ 22536. *A*: concentration-response relationship for G-1 in the presence or absence of PD98059, a selective inhibitor of MEK kinase. Results are expressed as means relaxation effect ± SE of 6 experiments. **P < 0.01, compared with the G-1 group by two way ANOVA analysis. *B*: A representative Western blot from 3 individual experiments of phospho-ERK1/2 detection in porcine coronary artery SMCs. Artery Cells were pretreated by in presence or absence of adenlyly cyclase inhibitor SQ22526, then incubated with DMSO (solvent control), G-1 (1 μM, with different collecting time points), G36 (10 μM) + G-1 (1 μM), and EGF (10ng/ml), a positive control. Protein amounts were normalized to β-actin, which was employed as a control for protein loading. *C*: Bar graph of the quantitative data of the Western blot bands evaluated by densitometry.

5.4 DISCUSSION

Although accumulative evidence suggests that GPER plays an important role in mediating cardiovascular actions of estrogen, our understanding of the mechanisms underlying these actions is extremely limited and sometimes contradictory. For example, a vasodilatory effect of GPER activation has been demonstrated in rat carotid (24), rat aorta (127), human mammary arteries (75), and porcine coronary arteries (144, 236); On the contrary, it has also been reported that GPER mediates a vasoconstrictor effect in

isolated perfused rat kidneys, and this constriction was blocked by inhibitors of EGFR and ERK1/2 (114). The mechanisms by which activation of GPER regulates coronary artery relaxation have been extensively investigated in chapter III and chapter IV. However, knowledge of GPER potentiation of coronary artery contraction in the cardiovascular system is lacking. This study demonstrated that activation of GPER induces EGFR transactivation and ERK1/2 activation via Gβγ signaling and thus promotes coronary artery contraction.

GPER is involved in estrogen potentiated aldosterone-angiotensin II-induced vasoconstriction in human coronary microarteries (12). Kurt et al. (114) further reported that G-1 induced vasoconstriction in basal renal perfusion pressure in the isolated rat kidneys. In contrast, G-1 induced vasodilation when the perfusion pressure is elevated by phenylephrine. However, no studies show a vasoconstriction effect of GPER on coronary arteries, which could contribute to estrogen-linked increased risk of heart attack in older women (130). We observed that G-1 (1 μM) enhanced the contraction effect of ET-1 in porcine coronary arteries, and G36 (10 μM), a selective GPER antagonist inhibited the action of G-1, suggesting that GPER activation mediates vasoconstriction as well as vasorelaxation.

After binding of GPER-specific ligands, Gα subunit in the activated trimetric G protein dissociates from the Gβγ complex and activates adenylate cyclase, resulting in the production of cAMP. The remaining Gβγ protein complex also acts as a signaling effector by activating Src tyrosine kinase, which binds to an adaptor protein shc. This complex activates membrane associated matrix metalloproteinase (MMP) and

subsequent release of EGF (epidermal growth factor) into extracellular space (177). EGF activates the EGFR (EGF receptor) through autocrine or paracrine to induce the activation of ERK1/2(57). Filardo et al. demonstrated that Gβγ protein complex mediated GPER downstream signaling in MCF-7 cells, SKBR3 cells and MDA-MB-231 cells (55). Our result that the selective Gβγ signaling inhibitor Gallein significantly increased G-1 induced coronary relaxation directly revealed the role of Gβγ in mediating coronary contraction effect of GPER activation.

EGFR transactivation plays an important role in vascular smooth muscle function and is likely involved in many cardiovascular diseases(100). Activation of EGFR has been implicated in blood pressure regulation, endothelial dysfunction, neointimal hyperplasia, atherogenesis, and cardiac remodeling (133). EGFR is involved in the mechanism of pressure-induced myogenic tone in mouse mesenteric resistance arteries (131). GPER-mediated renal artery constriction was inhibited by EGFR inhibitor, indicating involvement of EGFR in GPER-mediated regulation of vascular tone (114). However, no study has been found on how the modulation of GPER on EGFR signaling impacts coronary artery tone. Our results showed the functional role of EGFR in GPER-mediated potentiation of coronary artery constriction. The EGFR inhibitor AG1478 inhibited the contractile effect of GPER by increasing G-1 induced coronary artery relaxation. In Western blots, G-1 treatment increased the phosphorylation of EGFR in porcine coronary artery SMCs. Furthermore, in breast cancer cells, Src is upstream of EGFR transactivation mediated by GPER (55)**.** Src is involved in rat thoracic aorta contraction mediated by α1-adrenoceptors and in pressure-induced myogenic tone of

mouse mesenteric resistance arteries via transactivation of EGFR (131, 218). We employed PP2, the selective inhibitor of Src-family tyrosine kinases, to disrupt Src tyrosine kinase induced signaling and measured a cumulative ET-1 concentration-response relation in the presence or absence of G-1. PP2 reversed the enhanced contraction effect promoted by G-1, suggesting the functional role of Src. These experiments provided evidence for involvement of EGFR transactivation in the GPER potentiated coronary artery contraction.

The downstream of EGFR is the phosphorylation of ERK1/2, which has been implicated in vascular contraction by agonists of GPCR (G protein coupled receptors), such as ET-1 and growth factors (194). EGFR transactivation contributes to ET-1 induced vasoconstriction in rabbit basilar arteries via the ERK1/2 signaling pathway (102). Activation of ERK1/2 increased vasoconstriction in hemorrhagic shock rats, while, selective inhibitor of both MEK1 and MEK2 U0126 inhibited vascular contraction (232), which is in consistent with our finding that MEK inhibitor PD 98059 attenuate G-1 induce coronary artery contraction, leading to more relaxation. Filardo et al. (56) demonstrated that estrogen decreased ERK1/2 activity via Gβγ-dependent transaction of EGFR and it was through GPER-mediated cAMP-dependent signaling in breast cancer cells, suggesting the inhibitory effect of cAMP on ERK1/2 activity. We observed that GPER activation stimulated the phosphorylation of ERK1/2 via inhibition of adenylate cyclase by adenylate cyclase inhibitor SQ 22536, while in the absence of SQ22536 GPER attenuated phosphorylation of ERK1/2, indicating that cAMP maybe function as a switch between vasorelaxation and vasoconstriction mediated by GPER.

In conclusion, we have shown that GPER potentiated porcine coronary contraction which involves transaction of EGFR and the phosphorylation of ERK1/2 via Gβγ signaling (Fig. 26). This may help unravel the mystery of how estrogen can both prevent and contribute to coronary heart diseases.

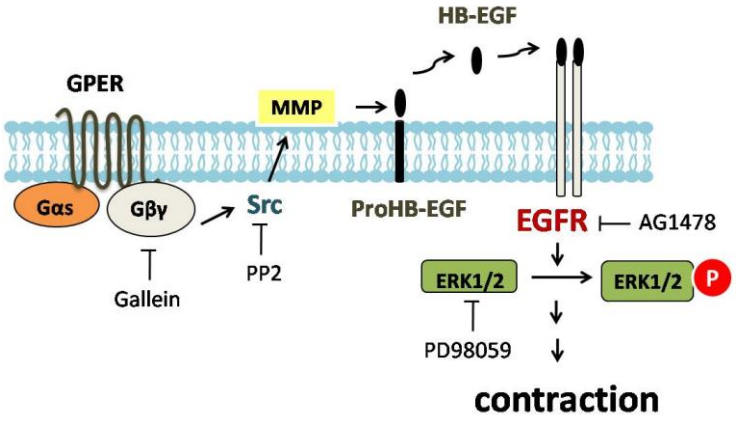

Figure 26. Proposed mechanism of GPER-enhanced porcine coronary artery contraction. When GPER is activated by agonist G-1, it activates the G protein Gβγ, which in turn stimulates tyrosine kinase Src, and then Src activates metalloproteinases which cleaves and releases the EGFR ligand precursors ProHB-EGF and causes transactivation of EGFR. Active EGFR stimulates its downstream target ERK1/2 and thus causes contraction.

CHAPTER VI

SUMMARY

6.1 CLINICAL IMPLICATIONS AND CONCLUSIONS

Coronary heart disease (CHD) is the #1 killer of women in the U.S., claiming the lives of over 500,000 women each year. Estrogen protects women from CHD, but estrogen levels decline after menopause and postmenopausal women are at particularly high risk for CHD. More recently, evidence showed the beneficial effects of postmenopausal estrogen therapy in younger postmenopausal women but more harmful effect to older postmenopausal women by increase the risk of heart attack and strokes. The hypothesis that activation of GPER induces opposing responses in porcine coronary artery function (a more human-like model of heart function) has been extensively investigated in this dissertation.

My work suggests that stimulation of GPER regulates porcine coronary artery tone by relaxing or contracting coronary arteries (Fig. 27). These dual effects of GPER may now help explain the paradoxical actions of estrogen on the cardiovascular system. My novel findings are that GPER activation: *1)* relaxes coronary arteries via activation of Gs protein and its downstream target PKA and Epac; *2)* through activation of MLCP via inhibition of RhoA activity by cAMP dependent stimulation of PKA in parallel with Epac; and *3)* induces coronary contraction by transactivation of EGFR and its downstream signaling. These two opposing signaling mechanisms provide fine tuning of coronary artery tone under healthy conditions.

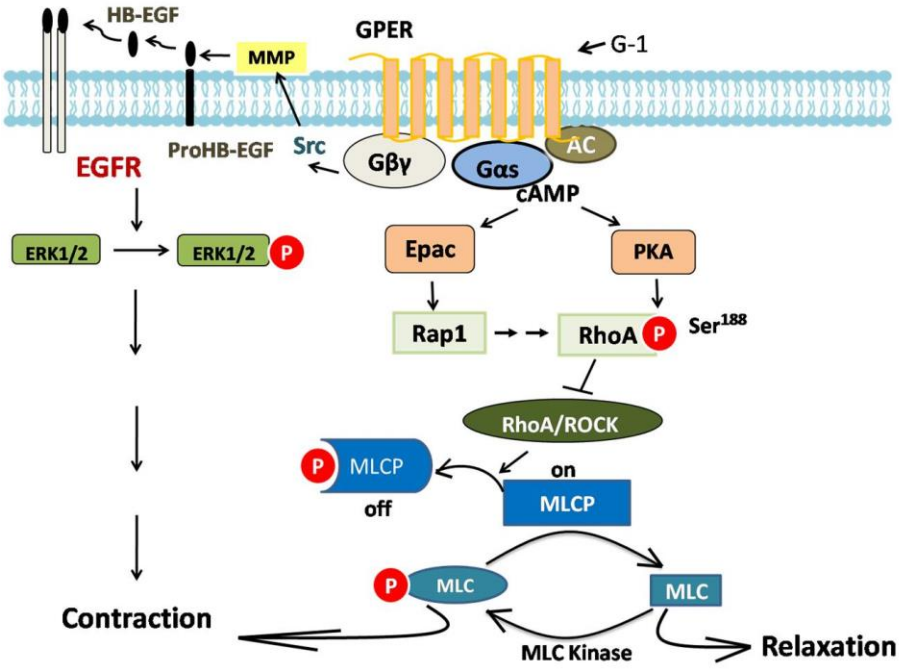

Figure 27. Proposed mechanisms of GPER dual actions on porcine coronary artery signaling. When GPER is activated by agonist G-1, it initiates both Gα/cAMP and Gβγ/EGFR signalings, and causes both vasorelaxation and vasoconstriction, with overall domination of vasorelaxation effect.

The mechanism by which activation of GPER induces coronary artery relaxation by a Gs/cAMP-dependent pathway or enhances contraction by a Gβγ/EGFR-dependent pathway is of clinical importance. It may provide some explanation regarding the controversial actions of how estrogen can both prevent and contribute to CHD, and thereby open significant opportunities for the development of new pharmacological strategies that would provide many health benefits of estrogen while limiting the potentially dangerous side effects. Further, if the therapeutic potential of estrogen could be understood and harnessed, novel therapeutic approaches could be developed that

would bring the quality health benefits of estrogen therapy to all people, without the endocrine side effects.

6.2 FUTURE STUDY

6.2.1 Relationship between Ca^{2+} modulation and BK_{Ca} channel activity

We observed that selective GPER agonist G-1 stimulates BK_{Ca} channel activity in both porcine and human coronary artery smooth muscle cells. Interestingly, G-1 is ineffective at stimulating BK_{Ca} channels in isolated excised membrane patches. These findings indicate that other regulatory mechanisms are involved in the opening of BK_{Ca} channel mediated GPER-induced relaxation. In vascular smooth muscle, an increase of BK_{Ca} channel activity from an elevation of cytoplasmic Ca^{2+} concentration consequently hyperpolarize the cell membrane to stop Ca^{2+} entry through L-type Ca^{2+} channels, decrease cytosolic $Ca^{2+}$and thus relaxes smooth muscle (213), suggesting a negative feedback between Ca^{2+} mobilization and BK_{Ca} channel activities. Further, activation of Epac induces a potent activation of BK_{Ca} channels in cerebellar granule cells via Rap/p38 MAPK/ Ca^{2+} mobilization (209). Therefore, to determine the relationship between Ca^{2+} mobilization and BK_{Ca} channel activities could be a future research direction.

6.2.2 Signaling related to AKAP

The study described in chapter III demonstrated that the inhibitor of AKAP (A-kinase anchor protein) -PKA interaction, FMP-API-1 (37), significantly inhibited G-1-

induced coronary relaxation and block the inhibitory effect of G-1 on the phosphorylation of MYPT-1 (the myosin light chain phosphate regulatory subunit), suggesting that the binding of PKA to AKAP is required for GPER mediated coronary vasorelaxation. Broselid et al. (23) showed that the C terminal of GPER interacted with membrane-associated guanylate kinases (MAGUKs) and AKAP5 (186), one of the AKAPS reportedly expressed in vascular smooth muscle cells, in plasma membrane. AKAP5 regulated cAMP signaling by taking PKA close to GPER through the coupling of AKAP5 and MAGUKS. These observations indicate that the plasma complex formed by GPER, MAGUKs and AKAP may function as an entity, and disruption in any part of the interaction may alter the downstream signaling. Therefore, the function role of this plasma complex needs to be investigated in future studies.

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